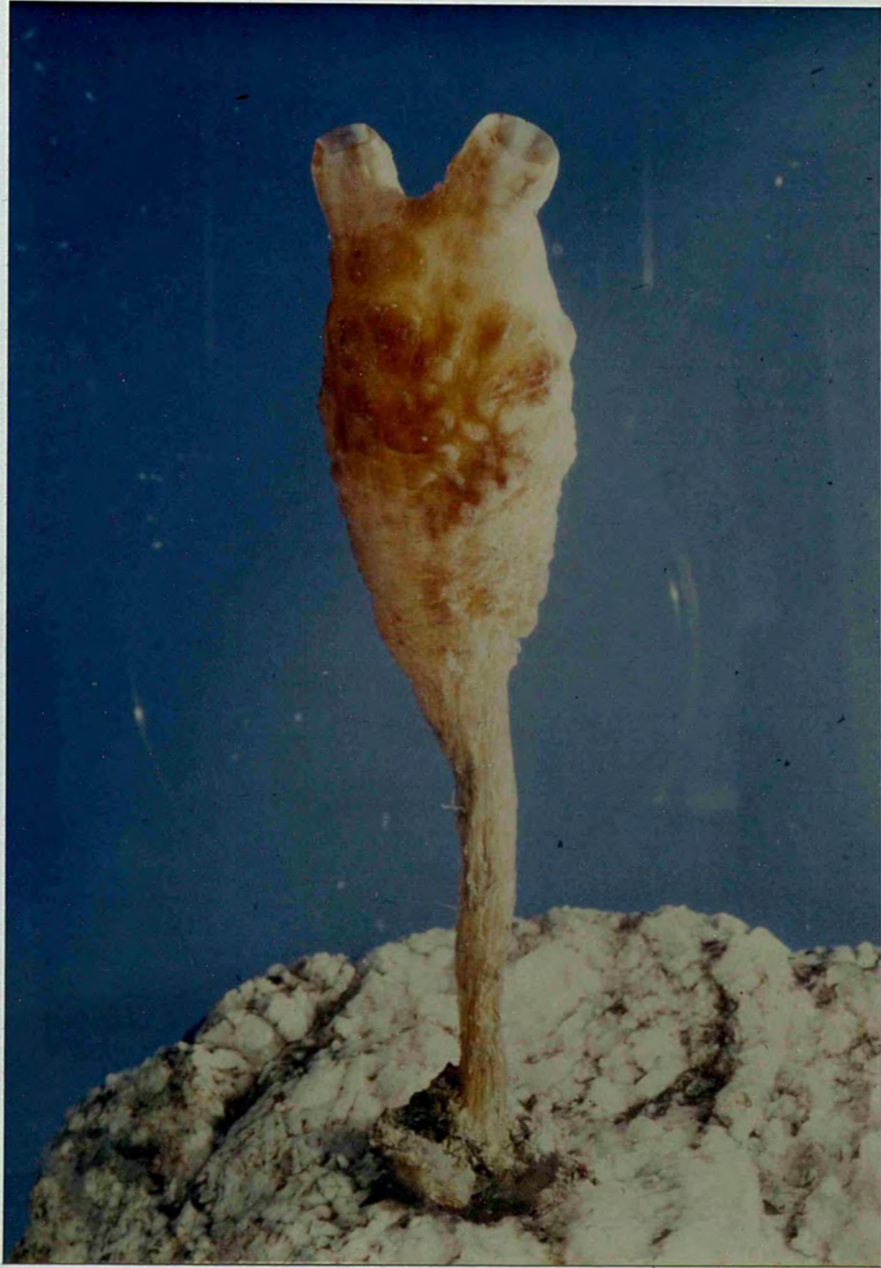

Styela clava

STUDIES ON GASTROINTESTINAL PEPTIDES IN THE ASCIDIAN

STYELA CLAVA

by

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Thesis submitted to the
University of London
for the degree of

Doctor of Philosophy

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ABSTRACT

Cells analogous to vertebrate endocrine cells have been described in the gut epithelium of the ascidian Styela clava. As well as some histochemical similarities, ultrastructural correlations have been demonstrated, particularly the presence of electron dense granules, clustered around and mainly below the nucleus, thinning out towards the apex. Like the endocrine cells of the vertebrate gut, the cells are often pyramidal, with a narrow apex which is occasionally observed to extend to the lumen of the gut.

In addition, strong secretin immunofluorescence was observed in the endocrine-like (E-L) cells of the mucous cap of the gastric ridges. Because of these observations, acid extracts of Styela gut were assayed for secretin in the rat and in the turkey. The Styela extracts as prepared were inactive but it is possible that this reflected faults in the extraction technique.

Development of a perfused Styela gut preparation, however, produced evidence to support the hypothesis that a CCK-like peptide is released into the circulation, presumably from the E-L cap cells, although CCK-like immunoreactivity is not demonstrable in these cells.

The observation that in addition to CCK, bombesin and physalaemin also induce enzyme release suggests that the pre-pancreatic zymogen cells contain a rich complement of receptors, corresponding to all the classes of PI stimulating receptors which have been found on vertebrate acinar cells. There is therefore the implication that these hormones or analogues may be present in Styela. As secretin was found not to act as a secretagogue in this system, the significance of its production is unclear. By analogy with vertebrate systems it may exert some control over the secretion of mucus by the cap cells.

CHAPTER 1 - LOCALISATION OF ENDOCRINE-LIKE CELLS IN THE GASTROINTESTINAL MUCOSA OF *STYELA CLAVA*

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The enterochromaffin cell

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The protochordate enterochromaffin cell

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INTRODUCTION

This chapter reports the finding of endocrine-like (E-L) cells in the gut of the ascidian Styela clava (Herdman, 1891) and their relationship to vertebrate gut endocrine cells. In order to do this it is first necessary to explain the nature of enterochromaffin cells, and the APUD theory of Pearse (1968a), that relates them to the other vertebrate endocrine cells.

The Enterochromaffin cell

The earliest description of cells which are now recognised as gastrointestinal endocrine cells was made by Heidenhain (1870), who reported that cells in the gastric mucosa of the dog and rabbit stained brown when fixed in bichromate solutions.

Cells similar to those of Heidenhain's were subsequently described independently by a number of authors. Many believed them to be their own discovery, and this has in consequence given rise to confusion concerning their nomenclature.

Nussbaum (1879) demonstrated granular cells in the stomach and pylorus after osmium fixation, and from his figures, these appear to be the same as Heidenhain's cells. Toldt (1880) also mentions granulated cells in an extensive paper on the development of gastric glands.

The first report of these granular cells in the intestinal epithelium was by Nicolas (1891) who fixed lizard intestine in Flemming's fluid, and after staining with safranine, saw occasional bottle-shaped cells, filled with small red granules. They were rediscovered by Kultschitzky (1897) in the canine intestine, noting that degranulation took place during fasting, an observation which was confirmed by Moller (1899).

In a brief paper, Ciaccio (1906) reported granulated cells in the canine crypts of Lieberkuhn, which stained with eosin, thionine blue, toluidine blue and iron haematoxylin. Ciaccio then published a far more detailed account of his cells (Ciaccio, 1907) attributing their discovery to Paneth in 1888 and mentioning the work of Nicolas, Moller and Kultschitsky. In this paper, Ciaccio described triangular or conical cells in dog and guinea pig intestine. He noted that they contained granules which could be stained yellow with potassium bichromate and because of this chromaffinity he considered they were similar to the cells of the adrenal medulla and suggested that they be called 'enterochromaffin cells'.

In a short paper, Kaufmann-Wolf (1911) noted the cells, and because of their characteristic appearance called them 'basalge kornte zellen', or basal granulated cells, and these were also noted by Kull (1913). Eklof (1914) in a monograph on the intestinal epithelium, made the unfortunate observation that he considered the granules to be fixation artefacts caused by coagulation of albuminoids into spheres.

Masson (1914) also described the enterochromaffin cells in human intestinal tumour tissue. He found that these cells could be stained with ammoniacal silver nitrate, using Bouin fixed tissue.

On the basis of these observations, Masson suggested that these cells should no longer be called enterochromaffin, as they could be confused with the adrenal chromaffin cells. Instead he suggested the epithet 'argentaffin'. Furthermore he made the inspired suggestion that these cells were endocrine and might constitute a diffuse gastrointestinal gland of endodermal origin, which was homologous with the pancreatic islets of Langerhans.

This considerably predates Feyter's (1938) concept of the diffuse epithelial endocrine organ, and perhaps Masson should be considered as the originator of the concept, albeit in a more simple form.

The argentaffin description was slow to gain acceptance. Kull (1925) described the cells as chromaffin, and Cordier (1926) as chromo-argentaffin. However the argentaffinity of these cells, ie their ability to reduce ammoniacal silver salts, was to become an important criterion for their identification.

Hamperl (1932) noted that some of the enterochromaffin cells were not argentaffin, but they were able to accumulate cytoplasmic silver deposits in the presence of reducing agents. These cells he described as argyrophilic. The two types of cells have also been called enterochromaffin (ie argentaffin) and enterochromaffin-like (ie argyrophil) (Pearse, 1974).

In 1938, Feyter suggested that the argentaffin and argyrophil cells of the gut might be considered as a 'diffuse epithelial endocrine organ'. The individual cells were considered to produce hormones that acted in a paracrine fashion on neighbouring cells and this theory laid the foundations for the development of the gastro-entero-pancreatic endocrine system and its unification by the APUD theory.

<u>The APUD theory</u>

Subsequent to work on the calcitonin producing parafollicular C-cells of the thyroid, Pearse (1966) called attention to a number of cells, including the enterochromaffin cell, which shared common cytochemical properties. This germ of an idea grew into the APUD theory (Pearse, 1968a), in which three sets of characteristics were used to define an APUD cell; ultrastructural, cytochemical and

TABLE 1 - Ultrastructural characteristics

1. Low levels of rough endoplasmic reticulum
2. High levels of smooth endoplasmic reticulum, as vesicles
3. High content of free ribosomes
4. Electron-dense, fixation-labile mitochondria
5. Prominent microtubules and centrosomes
6. Tendency to produce fine protein microfibrils (especially when neoplastic)
7. Membrane-bound secretion vesicles, with osmiophilic contents, best preserved by gluteraldehyde; varying density; average size 100-200 nm

embryological (tables 1,2,3).

The ultrastructural characteristics (Table 1) are fairly general, and are exhibited by most peptide secreting cells. The level of rough endoplasmic reticulum (RER), however, is dependent on whether the cell is actively synthesising its secretory product, in which case levels will be high, or storing it, in which case levels will be low. Pearse (1971) puts forward the proposal that the normal state of APUD cells is the storage phase as an explanation for the low levels of RER. But if an APUD cell is observed in a secretory phase, the RER levels can be elevated (Gould, 1978).

The high levels of smooth endoplasmic reticulum (SER) are certainly not an exclusive feature of APUD cells, as the limiting membranes of all secretory granules are presumably derived from endoplasmic reticulum. In this sense, any cell actively secreting granules, such as the acinar cells of the exocrine pancreas, could be considered to have high levels of SER.

The fourth and fifth characteristics are extremely vague, and this makes it difficult to regard them as clear cut APUD characteristics. In the case of the sixth characteristic, its relevance is limited by the fact that neoplastic cells are rarely observed except in medical school pathology departments.

The final characteristic is certainly a constant feature of APUD cells, though not unique to them. Because of the general nature of these ultrastructural features, it is clear that APUD cells cannot be identified with any certainty by electron microscopy alone, although it is a useful adjunct to other methods. The main use of the electron microscope in this field has been the classification of different types of cells on the basis of the size and shape of their secretion granules

TABLE 2 - Cytochemical characteristics

A)	1.	Fluorogenic amine content (catecholamine, 5-HT etc) or ability to secondarily take it up
P)		
U)	2.	Amine precursor uptake (5-HTP, DOPA)
D)	3.	Amino acid decarboxylase content
	4.	High side chain carboxyl or carboxyamide groups, sufficient for demonstration by masked metachromasia
	5.	High non-specific esterase and/or cholinesterase
	6.	High -GPD
	7.	Specific immunofluorescence

5-HT = 5-hydroxytryptamine; 5-HTP = 5-hydroxytryptophan;
DOPA = dihydroxyphenylalanine; -GPD = -glycerphosphate dehydrogenase
(after Pearse, 1969).

(Solcia et al, 1978a). However even this appears to require care, as granule appearance is easily altered by a number of fixation parameters (Mortensen and Morris, 1977).

The most constant cytochemical characteristics (Table 2) are 2,3,4 and 5, although if the first characteristic is present, then 2 and 3 may not be. The term APUD is an acronym derived from the initial letters of the first three characteristics, Amine and Precursor Uptake and Decarboxylation. APUD cells either contain a biogenic amine (ie 5-HT, domamine, epinephrine or norepinephrine) or are able to take it up. Also they are able to take up an amine precursor (ie 5-HTP or DOPA) and decarboxylate it (ie to 5-HT or dopamine).

The methods used for detecting amine content are based on fluorescence microscopy of formaldehyde fixed tissue. Enterochromaffin cells have been identified in this manner, using formalin fixed frozen tissue, since 1932 (Eros, 1932; Hamperl, 1932) but now formaldehyde vapour fixed, freeze-dried tissue is used after the method of Falck (Falck, 1962; Falck et al, 1962).

Gaseous formaldehyde reacts with biogenic monoamines such as 5-HT to produce compounds which fluoresce strongly in ultraviolet light. Amine precursor uptake can be demonstrated conveniently by intravenous injection of the L isomer of DOPA or 5-HTP. If tritiated DOPA or 5-HTP is used, then the resultant ^{3}H/-5-HT or ^{3}H/-dopamine can be visualised by autoradiography (Pearse and Ocumpaugh, 1966). If 'cold' amine precursors are employed, then the resulting amines can be visualised by the formaldehyde method of Falck.

Pearse had originally suggested that the process of peptide production may be linked to the uptake of 5-HTP and its subsequent decarboxylation to 5-HT (Pearse, 1966). It is now apparent that this

is the case, and all APUD cells demonstrate these amine handling properties. However other, non-endocrine protein producing cells have been shown to share these APUD properties of amine metabolism; notably cells from the exocrine pancreas (Alm, 1969; Alm et al, 1969) the chief cells (Håkanson et al, 1970) and Paneth cells of the stomach (Ahonen, 1973).

Some endocrine cells have been shown to stain with basic dyes such as pseudoisocyanin and toluidine blue (Epple, 1967; Solcia and Sampietro, 1965a; 1965b; 1965c). These basic dyes become attached to side-chain carboxyl groups of proteins (Solcia and Sampietro, 1965c) but are somewhat blocked in fixed tissue.

These side chains can be unmasked by acid hydrolysis (Solcia et al, 1968). Moreover, this technique converts side chain carboxyamido groups (glutamine and asparagine) to carboxyl groups (glutamic and aspartic acid). This characteristic is usually shown as 'masked metachromasia', with toluidine blue at pH 5.0 (Solcia et al, 1968) or the acridine dye, coriphosphine O (Bussolati et al, 1969).

The high levels of non-specific esterases and/or cholinesterases are a less specific feature of APUD cells. There is a considerable species variation, and high activity is often found in non-APUD cells (Hoyt et al, 1973; Pearse and Welbourn, 1973). A number of other techniques have also been employed to demonstrate APUD cells.

The lead haematoxylin method of Solcia (Solcia et al, 1969a) is also an effective staining technique for APUD cells. Like the basophilic and metachromatic stains it is thought to act on carboxyl groups, and is similarly made more effective by pre-treatment with HCl. However lead haematoxylin is more effective than the other two methods when the stains are considered without the HCl pre-treatment.

It would therefore appear that lead haematoxylin also shows some affinity for the carboxyamido groups that are unmasked by the acid hydrolysis. This method has also been adapted for use with semi-thin sections cut from resin embedded tissues (Gorgas and Bock, 1975).

Other useful stains, particularly for the gut endocrine cells are silver stains, which have been developed from the method of Masson (1914). These can be divided into two groups; those not requiring addition of a reducing agent, but relying on the cells containing endogenous agents able to reduce the silver ions in an ammoniacal silver salt solution (cf. Pearse, 1960), and those to which an exogenous reducing agent is added to cause the same precipitation of metallic silver to selectively impregnate the granules (Grimelius, 1968). In the first case the cells are regarded as argentaffin (or argentophil), and in the second case as argyrophil. The reducing agent present in the argentaffin cells is, in APUD cells, a biogenic amine such as 5-HT. This intrinsic reducing agent is not necessary when staining argyrophil cells, thus argyrophil cells are not necessarily argentaffin, but argentaffin cells are usually argyrophil.

Many APUD cells are stained by the argyrophilic Grimelius silver stain (Bussolati and Pearse, 1970; Grimelius, 1968; Solcia et al, 1969b; 1970), and it has even been adapted for staining resin sections for ultrastructural observation (Grimelius and Strand, 1974; Vassallo et al, 1971).

The final cytochemical characteristic of APUD cells, as given by Pearse, is the specific immunocytochemical localisation of the peptide that they produce. These have been determined for most of the postulated APUD cells, and are shown in table 4. The specific demonstration of the peptide product is perhaps the most important test

TABLE 3

1. Although the cells have diverse origins, they have evolved similar biochemical mechanisms for the production of similar peptide products.

2. Although the cells have diverse origins, they have evolved similar biochemical mechanisms in response to similar specific secretory stimuli (aminergic or cholinergic).

3. They have a common embryologic ancester, and have retained a common set of ancestral functions.

(after Pearse, 1969)

TABLE 4 - THE DIFFUSE NEUROENDOCRINE SYSTEM

CENTRAL DIVISION

Cell type	Peptide product	Amine product(s)
Pineal	Arginine vasotocin, lutropin releasing hormone	Melatonin 5-HT
Hypothalamic magnocellular	Arginine vasopressin, arginine vasotocin	5-HT Dopamine Norepinephrine
Hypothalamic parvocellular	Releasing factors, release inhibiting factors	
Pituitary pars distalis	FSH, LH, TSH STH, PRL, ACTH MSH, -LPH Endorphin Gastrin, calcitonin	5-HT Dopamine Norepinephrine
Pituitary pars intermedia	ACTH, MSH LPH Endorphin, calcitonin	Histamine Tyramine

(The amine products 5-HT, Dopamine and Norepinephrine are bracketed jointly for the hypothalamic magnocellular and parvocellular cells.)

PERIPHERAL DIVISION

Cell type		Peptide product	Amine product(s)
Pancreas	B,A	Insulin, glucagon	5-HT
	D, D_2	Somatostatin, VIP,PP	Dopamine
Stomach	G,	Gastrin, ENKEPH, ACTH	
	AL,	Glucagon	
	EC_1	Substance P	5-HT
	ECL		Histamine
	D	Somatostatin	
Intestine	EC_1	Substance P	5-HT Melatonin
	EC_2(M)	Motilin	
	L,S,	Glicentin, Secretin	
	I,D(P)	CCK, Bombesin	
	D.K,	Somatostatin, GIP	
	N,	Neurotensin	
	H	VIP	
Lung	K	Bombesin	
Parathyroid	Chief	Parathyrin	
Adrenomedullary	E,NE		Epinephrein, norepinephrine
Sympathetic Ganglion	VIP		Norepinephrine
	SIF		Dopamine, Norepinephrine
Carotid body	Type 1	ENKEPH	Dopamine, Norepinephrine
Melanoblast/cyte			Promelanin
Thyroid/ ultimobranchial	C	Calcitonin, somatostatin	5-HT
Urogenital tract	EC U		

Abbreviations: PP = pancreatic polypeptide; EC = enterochromaffin; ECL = enterochromaffin-like; ENKEPH = Met-or Leu-enkephalin; SIF = small intensely fluorescent, Glicentin formerly enteroglucagon; 5-HT = 5-hydroxytryptamine

(after Pearse and Takor Takor, 1979)

of all, as only then can the endocrine nature of the product be experimentally determined.

Embryology

Since Pearse (1969) considered it unlikely that the similarities, in the ultrastructure and cytochemistry of the diverse cell types in the APUD series, were coincidental, he advanced three hypotheses which might explain their common features (Table 3).

The first two hypotheses would be difficult to test experimentally but the third has received considerable attention since it was proposed. Debate and experimentation has centred around the question - 'which is the common embryological precursor cell?'

When Pearse (1968a) first proposed the APUD series he was of the opinion that all the cells had an endodermal origin, as all the cells then being considered were in the gut, or in glands or tissues derived from the primitive digestive tube. By the following year however (Pearse, 1969) his view had changed and without reference to the earlier view he stated that the only possible APUD ancestor was the (ectodermal) neural crest cell. Experiments over the next few years, principally by Pearse and the Le Douarin group (Le Douarin and Le Lievre, 1971; Le Douarin et al, 1972; Pearse and Polak, 1971; Pearse et al, 1973) caused a slight revision to the neural crest hypothesis. Pearse and Takor Takor (1976) broadened the concept of a neural crest origin of all APUD cells, to include the neural crest, neural ridges, neural tube and specialised placodal ectoderm, all of which are neuroectodermal.

Although a considerable body of evidence has been produced by Pearse to favour this theory (Pearse and Polak, 1971a; 1971b; 1971c;

Pearse et al, 1973a; Pearse and Takor Takor, 1976; Takor Takor and Pearse, 1975), the results of experiments performed by other workers have not always been in agreement.

It is almost certain that the adrenal chromaffin cells are neural crest derivatives (Hamilton et al, 1972; Le Douarin and Teillet, 1971; Polak et al, 1971; Weston, 1970) as is also the case for the melanoblasts (Teillet and Le Douarin, 1979). The calcitonin producing C cells and the carotid body type 1 cells have also been shown to be derived from neural crest, at least in birds, by a series of allograft experiments, using chick/quail chimerae, by the Le Douarin group (Le Douarin and Le Lievre, 1971; Le Douarin et al, 1972). Cytochemical studies by Pearse and Polak (1971a) and Pearse et al, (1973b) have confirmed this finding. The evidence for a neuroectodermal origin for other APUD cells is less certain, and more contentious. To evaluate all the experimental evidence concerning the embyrological derivation of all these cell types lies outside the scope of this thesis, but the origin of the gut endocrine cells will be considered as being more relevant.

The evidence against the enterochromaffin cells of the gut having a neural crest origin is quite conclusive (Andrew et al, 1983). Andrew (1963; 1974; 1975) conducted a series of experiments in which she removed the presumptive gut from 10-somite chicks, ie before the presumed arrival of neural crest cells. However, when these presumptive gut cells were cultured, enterochromaffin cells still occurred in the intestine. Le Douarin and Teillet (1973) using chick/quail chimerae have also provided evidence suggesting that the gut enterochromaffin cells are not of neural crest origin. Pearse and Takor Takor (1976) suggested that these results were invalid, as they rely on the assumption that the neural crest has been removed before

its cells have migrated. Their studies suggest that this takes place in birds at the 6-somite stage, which is long before the extirpation experiments of Andrew (1963; 1974; 1975) and the allograft experiments of Le Douarin (Le Douarin and Teillet, 1971; 1973) were carried out.

Lamers et al (1981) have worked on embryos of the cyprinid fish Barbus conchonius which had been injected with labelled neural crest cells. They subsequently found no neural crest derivatives in the gut epithelium.

Andrew (Andrew et al, 1980; 1982) has subsequently carried out further experiments using techniques similar to those of Le Douarin, where endoderm and mesoderm from chick and quail were combined, neural crest cells being excluded. These explants, after culture, were tested by electron microscopy and immunocytochemistry and a broad range of endocrine cells were found to have developed. They conclude that 'the majority, if not all, gut endocrine cell types arise from endoderm, and not mesoderm or ectoderm'. On the evidence available, this conclusion seems likely to be the correct one.

The protochordate enterochromaffin cell

In 1970, Winbladh Biuw and Hulting (1971) noted that some of the mammalian gut hormones had been found in lower vertebrates and invertebrates. Because of the links of the protochordates with the ancestral chordates (Barrington, 1965), they studied the gut of the lancelet, Branchiostoma lanceolatum, in the hope of finding cells homologous with the vertebrate enterochromaffin cells, or the pancreatic endocrine cells. Finely granulated, apparently secretory, cells which had staining reactions similar to the insulin producing β cells of the vertebrate endocrine pancreas were found.

Following this initial description, Kataoka and Fujita (1974) noted that on the basis of granule morphology there were in fact at least two types of endocrine cells in the lancelet, and they drew attention to the ultrastructural similarities between these and the vertebrate, basal granulated enterochromaffin cells.

These results were quickly confirmed by Van Noorden and Pearse (1976). They located the two granulated cells of Kataoka and Fujita, and also found two other cell types with a similar endocrine-like ultrastructural appearance. They further demonstrated that these cells showed staining reactions similar to cells of the vertebrate gastro-entero-pancreatic endocrine system. Using antisera raised against vertebrate gut hormones they showed cells which appeared to be producing glucacon-, gastrin- and insulin-like peptides.

At the same time, similar reports were also being made concerning E-L cells in another group of protochordates, the Ascidiacea; Lison (1933) had described enterochromaffin cells in the stomach of Ciona intestinalis, and Gerzeli (1963; 1964) had found similar cells in a wide range of ascidians. Ultrastructural confirmation was now provided by Burighel and Milanesi (1975). These later authors noted cells in the gastric epithelium of the ascidian Botryllus schlosseri which were ultrastructurally similar to endocrine cells of the vertebrate gut. Fritsch (1976) noted argyrophil and argentaffin cells in the gut of Ciona, and the following year, Fritsch and Sprang (1977) studied their ultrastructure and considered it to be typical of mammalian gut endocrine cells.

Thorndyke (1977) also reported cells within the mucous cap region of the gastric ridges of Styela clava which were ultrastructurally endocrine-like. The present study was initiated as a direct consequence

of this observation.

SECTION 1 - HISTOCHEMISTRY

MATERIALS AND METHODS

Adult specimens of *Styela clava* were collected in the Portsmouth area from pontoons sited about one kilometre offshore. These were then transported to London by van in aerated sea water. Animals used for histological work were processed immediately. Although some animals were maintained in a circulating sea water aquarium at 10° to 14°C, these were not used for histological examination.

The animals were rapidly dissected and portions of oesophagus, stomach and intestine were treated in one of a number of ways.

Some tissues were fixed for 24 hrs in Bouin's fluid that had been prepared using picric acid saturated sea water. Other tissues were fixed overnight with 6% glutaraldehyde (glutaric dialdehyde) in phosphate buffer at pH 7.0 (appendix 1). This material was dehydrated in graded ethanols, cleared in xylene and embedded in 56°C mp paraffin wax.

For the demonstration of biogenic monoamines after the method of Falck et al (1962), small pieces of tissue were quenched in melting Arcton (mp -148°C) which had been pre-cooled in liquid nitrogen. These were then freeze dried using an Edwards EPTD2 tissue drier. Following subsequent fixation over paraformaldehyde (ie in formaldehyde vapour) for 3 hrs at 60°C the tissue was embedded in 56°C mp paraffin wax in a vacuum oven.

Sections were cut at 6 to 8 μ on a Jung rotary microtome. These were subsequently stained by one of the following methods; argyrophilia (Grimelius, 1968), argentaffinity (Solcia et al, 1969b), masked metachromasia (Solcia et al, 1968), lead haematoxylin (Solcia et al, 1969a) or aldehyde fuchsin (Bussolati and Bassa, 1974). Full details of these staining methods are given in appendix 1.

RESULTS

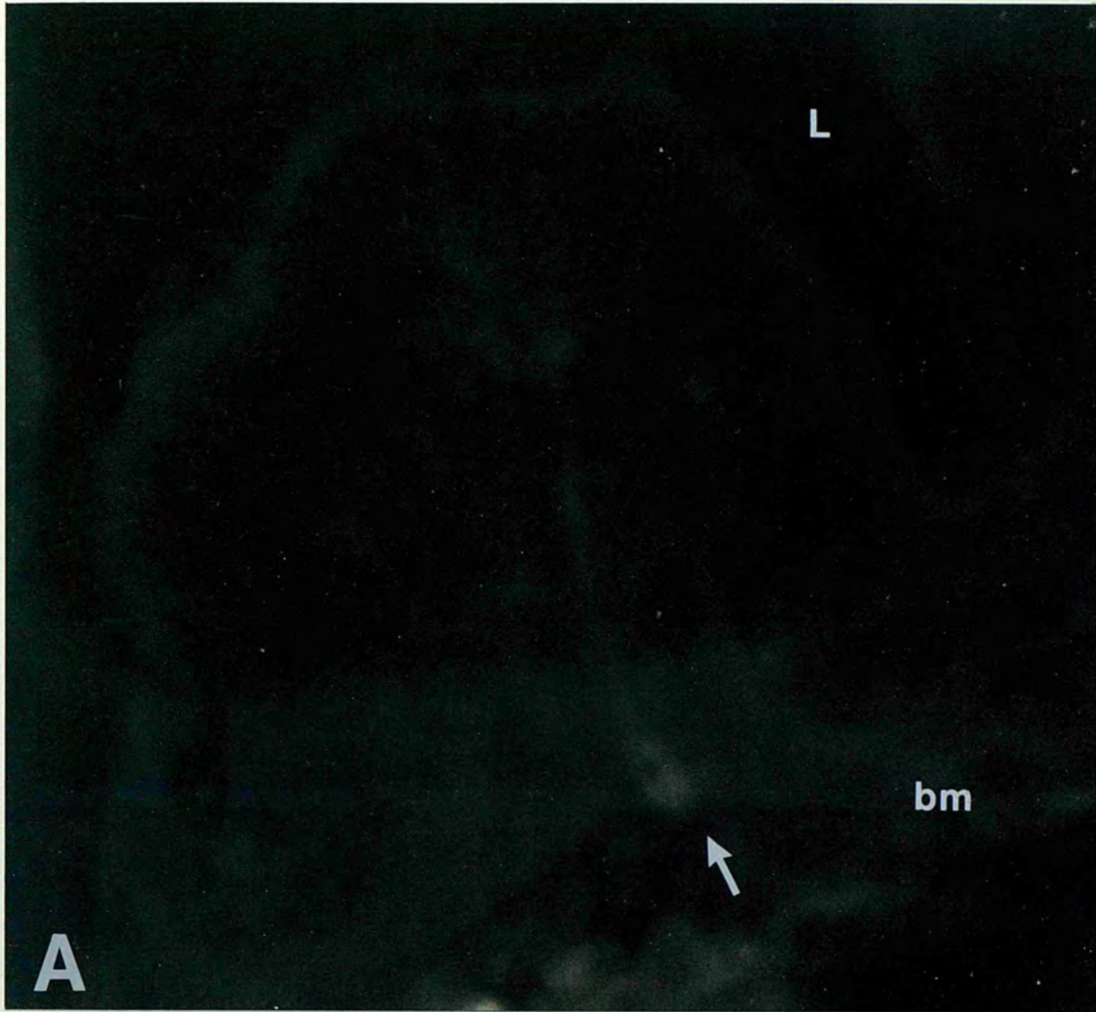
Oesophagus

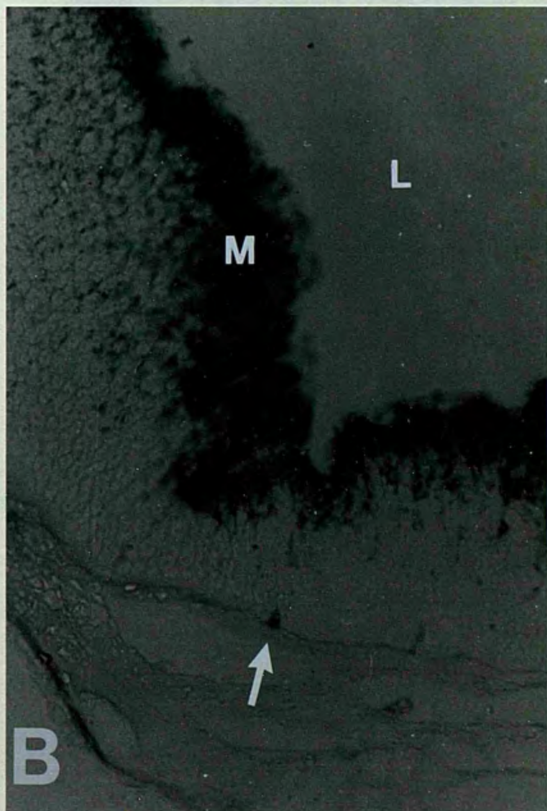
The staining reactions of the oesophageal endocrine-like cells in *Styela* are somewhat capricious. The reason for this is not clear, but as the stains are only weakly taken up when staining does occur it is probably caused by a weak affinity between the cells and the stains employed.

Aldehyde fuchsin was the most successful and reproducible stain. Here, small granulated cells were observed lying on the basement membrane (Fig 1:1b). The dense population of oesophageal mucous cells was also stained, but as only the apical region, adjacent to the gut lumen, was stained, they did not interfere with the observation of cells on the basal lamina. Grimelius' method for argyrophilia also gave positive results showing cells containing distinct granules (Fig 1:1c). When staining was successful, considerably fewer cells were stained than by aldehyde fuchsin, but they would be seen to occupy a similar position in the epithelium, on the basal lamina.

No staining was observed using techniques for argentaffinity, masked metachromasia or lead haematoxylin.

Fig 1.1 Light photomicrographs showing oesophageal epithelium of Styela clava. a) Insulin-immunoreactive cell (arrow) following immunofluorescence staining with antiserum to insulin (1:10), x 1750. b) Aldehyde fuchsin stained cell (arrow) resting on the basement membrane, x 650. c) Argyrophilic cell (arrow) showing distinct granules clustered around the unstained nucleus, x 1500. BM = basement membrane; L = lumen; M = apex of mucous cell.


L
bm
A


M
L
B

C

Fig 1:2

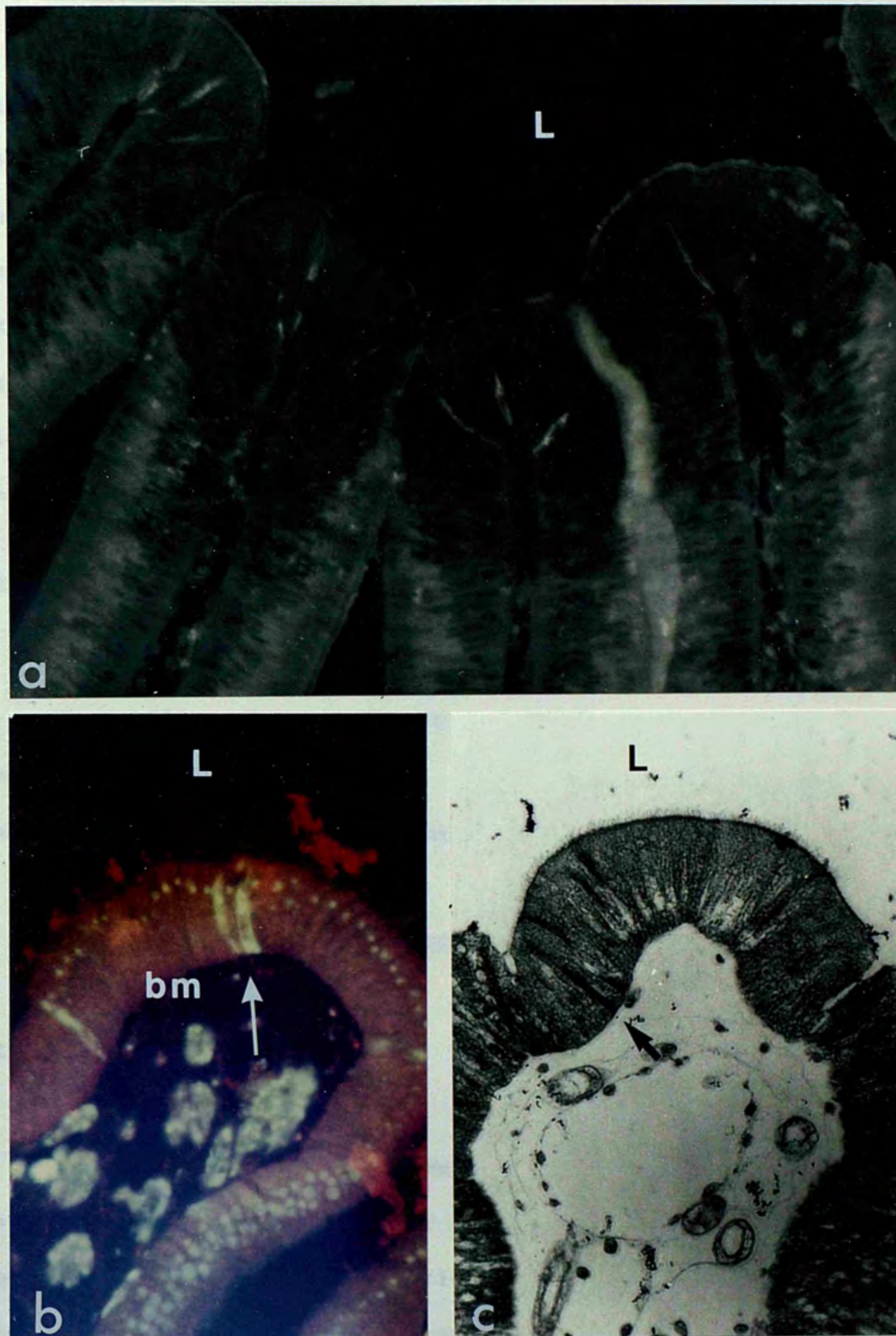


Fig 1:2 a-c: Light photomicrographs of the cap region from the gastric epithelium of Styela clava. a) Immunofluorescence of cells after treatment with dilute anti-secretin serum, A (1:5000), x 200. b) Formaldehyde induced fluorescence, x 500. c) Lead haematoxylin, x 500. L = lumen; BM = basement membrane.

Stomach

The gastric epithelium of Styela clava consists of 20 to 30 longitudinal folds, together with a single, flat strip or raphe of cells. At the apex of each ridge is a distinct 'cap' of ciliated mucous cells. Within these caps are a number of elongated cells, extending from the basement membrane to the luminal edge, which stain weakly with lead haematoxylin (Fig 1:2a), and exhibit formaldehyde induced fluorescence (FIF) (Fig 1:2b). However these cells did not stain with methods for argyrophilia, argentaffinity, masked metachromasia or aldehyde fuchsin.

Intestine

No cells were stained in the intestinal epithelium using methods for argyrophilia, argentaffinity, masked metachromasia, lead haematoxylin or aldehyde fuchsin. The intestinal epithelium was not examined using methods to demonstrate FIF.

SECTION 2 - ELECTRON MICROSCOPY

MATERIALS AND METHODS

Before observing cell structures, it is necessary to preserve them with the introduction of minimal artefacts. Conventional fixation normally involves the immobilisation of the structural proteins by cross-linking them, normally with an aldehyde such as formaldehyde or glutaraldehyde. Glutaraldehyde is particularly good and is normally considered to be the fixative of choice for electron microscopy. Its main disadvantage is that its action is slow and its penetrative power is low. It is only possible to fix very small pieces of tissue (1 mm^3)

and fixation takes at least 2 hr (at 4°C). This primary fixation is normally followed by a period of post-fixation with 1% osmium tetroxide, which cross-links between tissue proteins and unsaturated lipids and also helps reduce lipid loss during dehydration (Nielson and Griffith, 1979).

The choice of fixative is however only one variable. To obtain optimal fixation and to enable comparisons to be made of ultrastructural appearance between tissues fixed on different occasions, it is essential to rigorously maintain all the other variables (choice of buffer, pH, duration, temperature and osmolarity).

One of the most important of these is osmolarity. For glutaraldehyde fixation the vehicle must be close to, or slightly below, the osmolarity of the extracellular fluid (Bone and Ryan, 1972). In the case of an osmoconformer such as Styela this is isosmotic with seawater (c. 1030 mOsm/kg). This was achieved by adding filtered artificial seawater to the basic 3% cacodylate buffered glutaraldehyde.

Small pieces of gut (approx 1 mm^3) from animals freshly collected from Portsmouth, were rapidly excised into cold (4°C) 3% glutaraldehyde (glutaric dialdehyde), in 0.1M sodium cacodylate buffer (pH 7.2) in sea water. Further details of this 'marine fixative' are given in appendix 1.

Tissues were fixed for 2 hrs in sodium cacodylate buffer and post-fixed for 1 hr in cold (4°C) 1% osmium tetroxide prepared in cacodylate buffer (pH 7.2). Following fixation, the tissue was washed in buffer overnight and transferred by graded ethanols and propylene oxide (1,2-epoxypropane) to TAAB laboratories epoxy resin. These

preparations were then polymerised for 48 hrs at 60°C (for full schedule, see appendix 1).

Thin sections were cut, using glass knives, on a Cambridge Huxley II ultramicrotome. Tissue orientation was facilitated by taking thick (0.5μ) sections which were mounted onto glass slides by gentle heating, and stained with 1% toluidine blue in borax (appendix 1). Thin sections showing silver or pale gold interference colours were then picked up on uncoated copper or nickel grids. These were then stained in a freshly filtered saturated solution of uranyl acetate in 30% ethanol for 20 mins and then after rinsing with distilled water, were counterstained for 5 to 10 mins in alkaline lead citrate (appendix 1). After washing in 0.02 M sodium hydroxide and distilled water, the grids were allowed to dry and were then examined on a Corinth 275 or Zeiss 109 electron microscope.

RESULTS

Granulated cells, ultrastructurally similar in appearance to vertebrate gastrointestinal endocrine cells, were found at all levels of the post-pharangeal digestive epithelium of Styela, although the overwhelming majority were found in the gastric epithelium.

Oesophagus

The oesophageal E-L cells were distributed evenly, but sparsely amongst the mucous cells which form the oesophageal mucosa (Fig 1:4-1:6). They occurred on the basal lamina and although they occasionally appeared slightly elongated (Fig 1:4), they were not observed to extend to the gut lumen and were therefore presumed to be of the closed type (Fig 1:3).

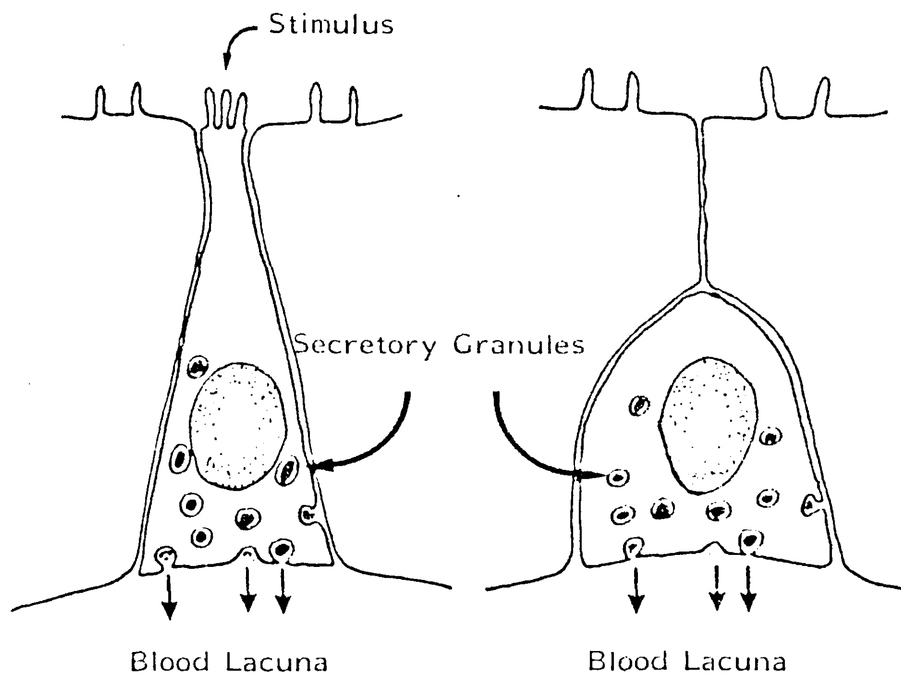
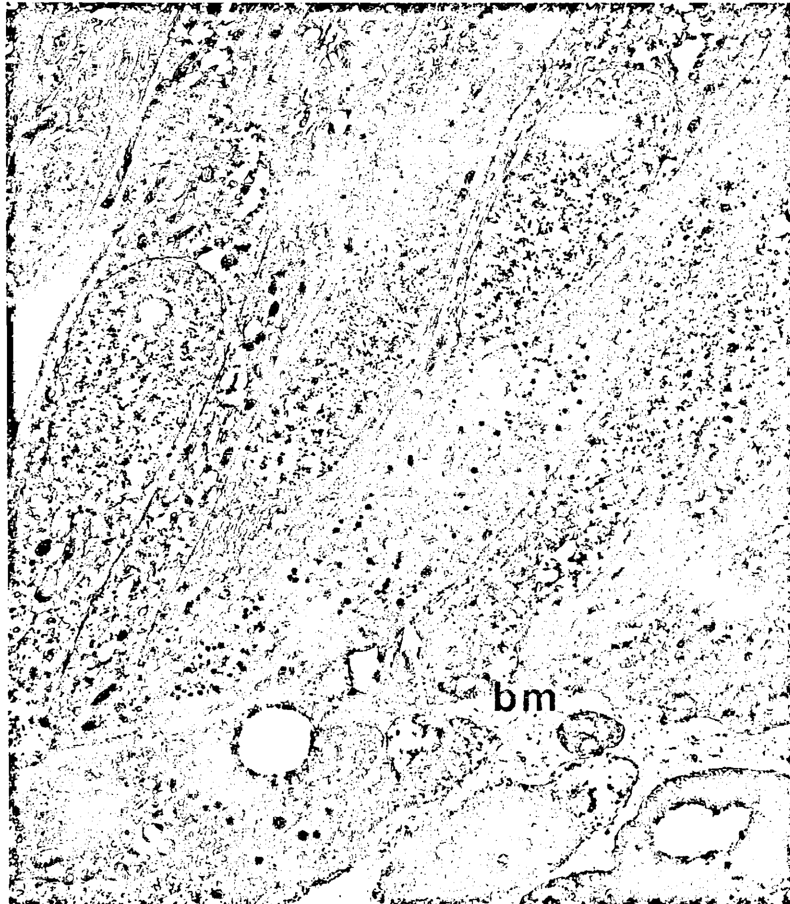


Fig 1:3 Endocrine cells of open (left) and closed (right) type, observed in the gastric and oesophageal epithelium respectively. Open cells reach the lumen via an apical process equipped with microvilli, whereas closed cells do not reach the lumen. Secretory products are presumed to be released by exocytosis.

Fig 1:4 A medium power electron photomicrograph showing a typical granulated oesophageal E-L cell (arrow), extending a short distance from the basement membrane and flanked by oesophageal mucous cells.

bm = basement membrane (x 8000)

Fig 1.4



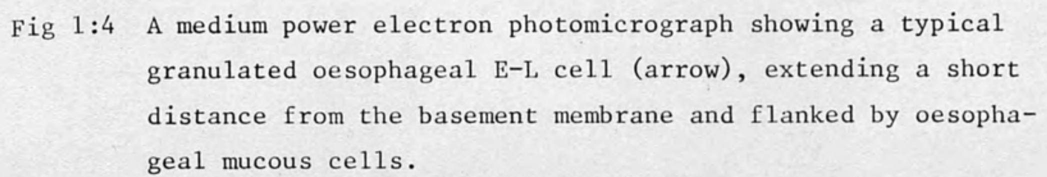


Fig 1:4 A medium power electron photomicrograph showing a typical granulated oesophageal E-L cell (arrow), extending a short distance from the basement membrane and flanked by oesophageal mucous cells.
bm = basement membrane (x 8000)

Fig 1.4

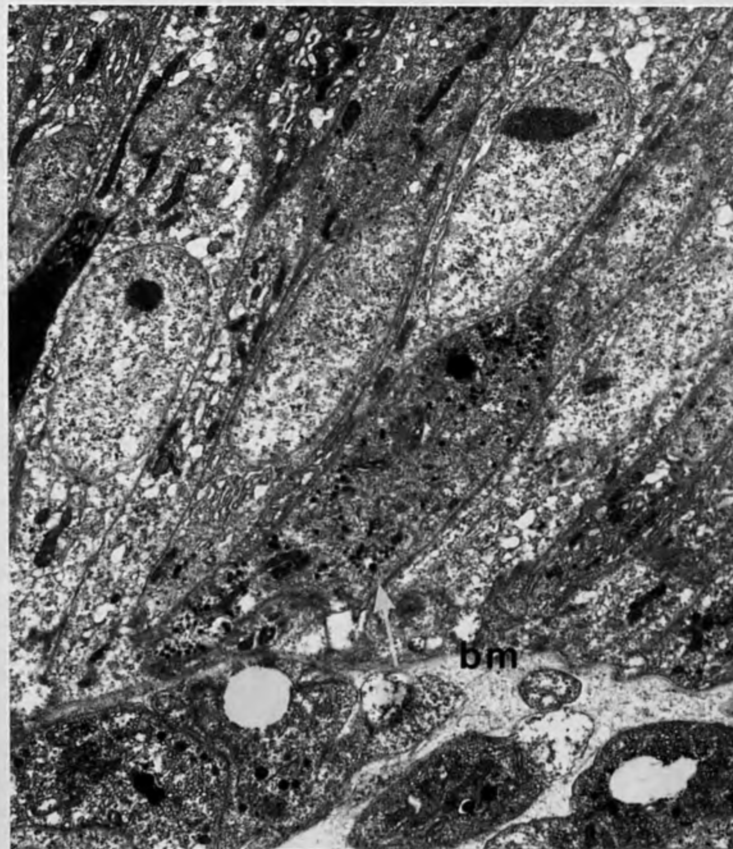


Fig 1:5 a) A low power and b) a medium power electron photomicrograph, showing the same oesophageal E-L cell (arrow), including a section of the nucleus. This cell, typically, is not observed to extend to the oesophageal lumen. The majority of the regularly shaped, electron-dense granules can be observed to have a very narrow, or non-existent halo, to vary little in size, and to be concentrated in the subnuclear region. Mitochondria are present throughout the cytoplasm. There is evidence of the exocytotic release of these granules at the basal lamina (fig b, arrows).
L = lumen; m = mitochondria; n = nucleus (a, x 5000; b, x 13250)

L
n
A

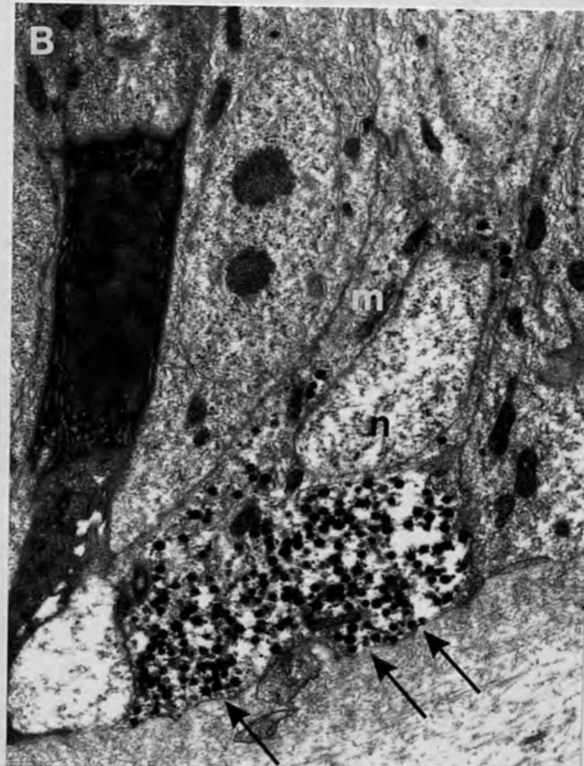
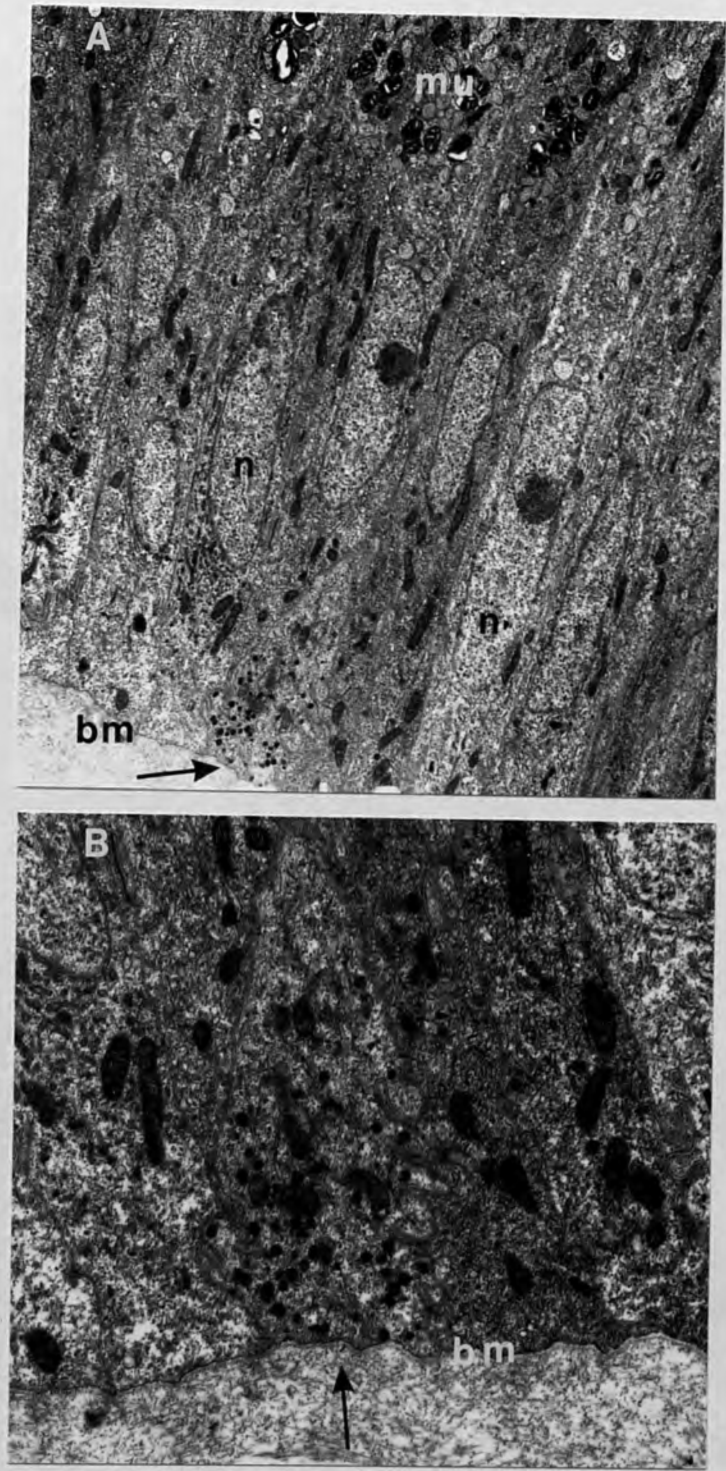

B
m
n

Fig 1:6 a) A low power and b) a medium power electron photomicrograph showing an oesophageal E-L cell (arrow) extending a short distance from the basement membrane. The other (mucous) cells can clearly be seen. They are tall and narrow with an apical mass of mucus. The large nuclei fill most of the width of the cells and are sited in the basal region. The nucleus of the E-L cell is, as is common, not included in this section.

bm = basement membrane; mu = mucus; n = nucleus

(a, x 6000; b, x 13000)

Fig 1.6



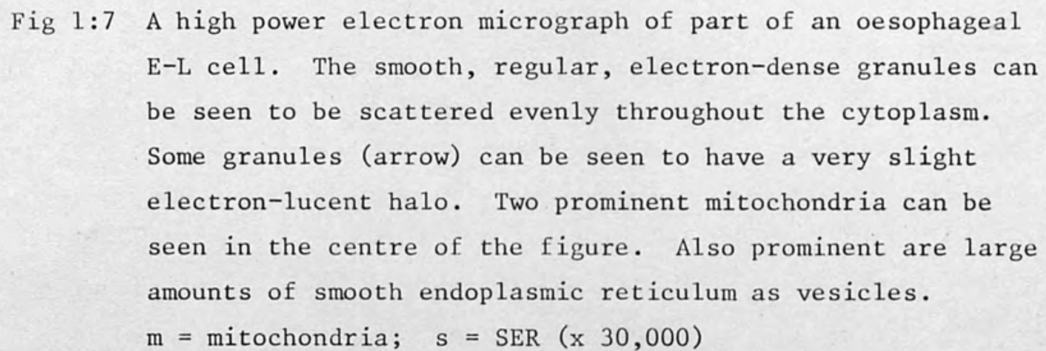
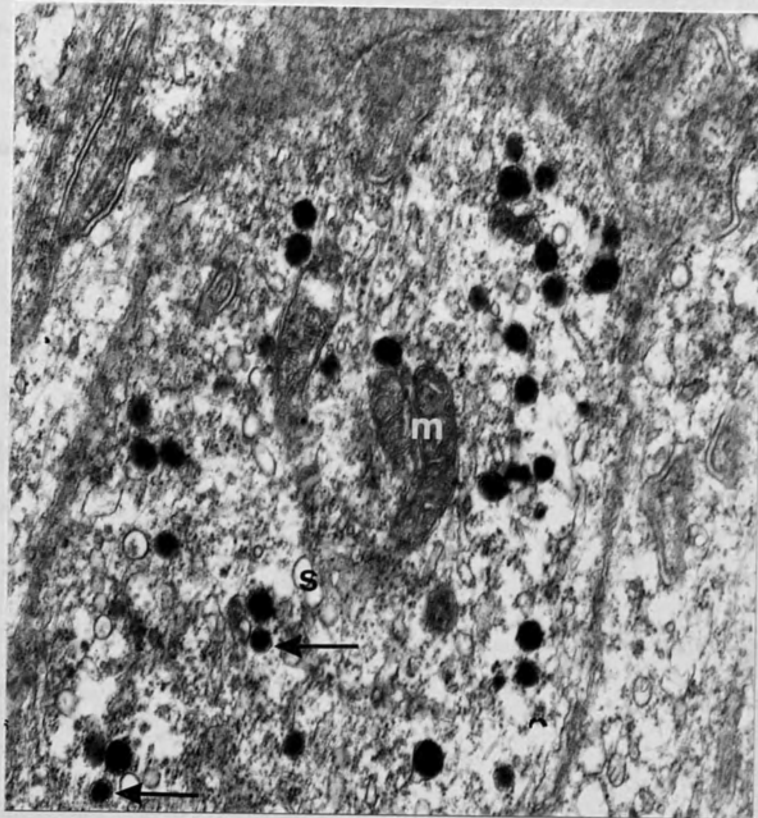


Fig 1:7 A high power electron micrograph of part of an oesophageal E-L cell. The smooth, regular, electron-dense granules can be seen to be scattered evenly throughout the cytoplasm. Some granules (arrow) can be seen to have a very slight electron-lucent halo. Two prominent mitochondria can be seen in the centre of the figure. Also prominent are large amounts of smooth endoplasmic reticulum as vesicles. m = mitochondria; s = SER (x 30,000)

Fig 1.7



The granules were regular in shape with a narrow or non-existent halo. They varied only a little in size (100 to 175 nm, av 130 nm diameter) and gave every appearance of being an homogenous population.

In sections where the nucleus was included (Fig 1:5) very few supra-nuclear granules were observed, the majority being grouped between the nucleus and the basement membrane.

Rough endoplasmic reticulum was not observed, but smooth vesicular endoplasmic reticulum was often prominent (Fig 1:7). Golgi membranes were only occasionally seen but mitochondria were present in all sections.

Stomach

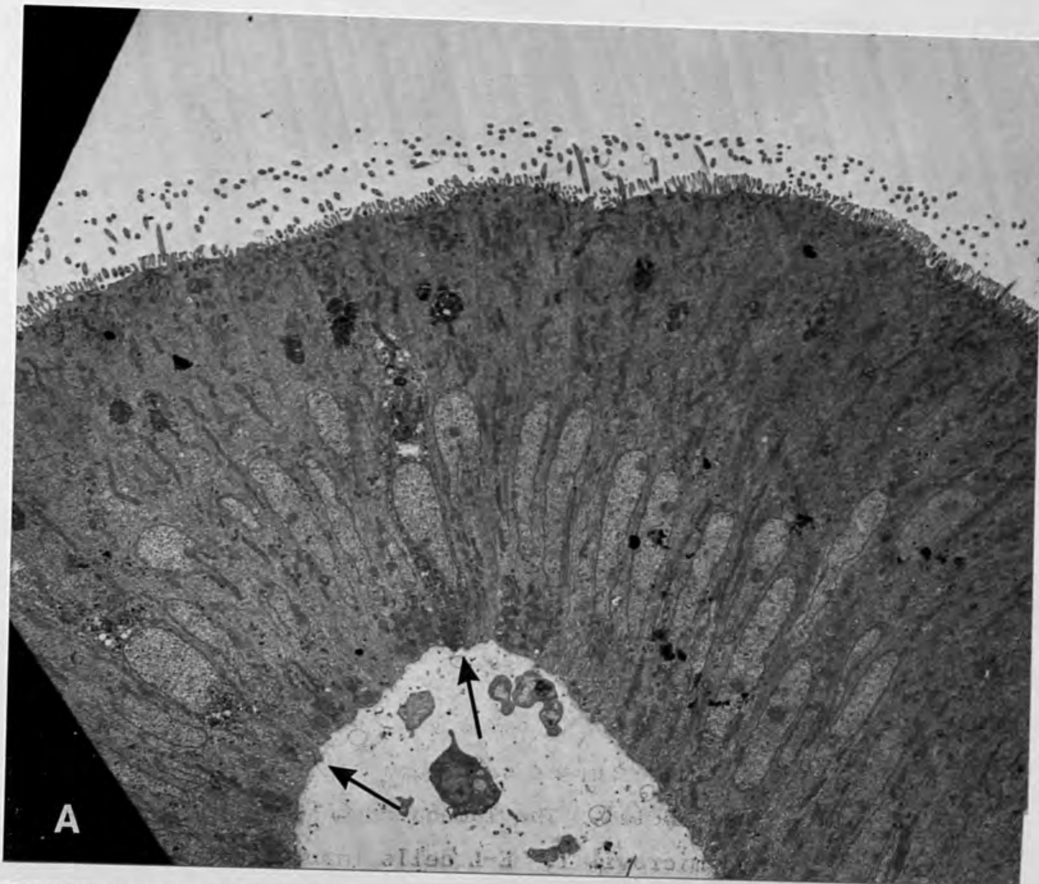
The gastric E-L cells also extended from a position on the basal lamina but unlike the oesophageal population, they were frequently observed to extend to the luminal edge (Fig 1:8). They were observed to lie only in the mucous 'cap' region of the gastric ridges. No E-L cell of any description was observed in the side walls. The overall shape of these E-L 'cap' cells was columnar, but slightly tapered so that the apex was narrower than the base.

The juxtaluminal part of a gastric E-L cell was readily distinguishable from the apices of the flanking mucous cells, which contained numerous mitochondria and obviously secretory granules. In the E-L cells there were few or no apical granules nor mitochondria, and therefore no indication of luminal secretion. The cells reach the lumen in a frequently constricted region which was tipped with a tuft of microvilli (Fig 1:9).

The rounded nucleus lay in the lower mid-zone of the cell. The secretory granules were clustered below and around the nucleus and

Fig 1:8 a) A low power and b) a high power electron photomicrograph of the mucous 'cap' region of one of the gastric ridges.
a) This plate shows the major constituent cells of the mucous cap. These mucous cells are tall, with large basally sited nuclei. The lumenal membrane bears a number of cilia and microvilli. E-L cells (arrows) of similar proportions, appear scattered throughout the 'caps'.
b) An enlargement of of the basal part of the central E-L cell from (a), showing the typical irregular appearance of the electron-dense granules, and their surrounding electron lucent halos.
bm = basement membrane; g = granule; m = mitochondria; n = nucleus. (a, x 2700; b, x 30,000)

Fig 1.8



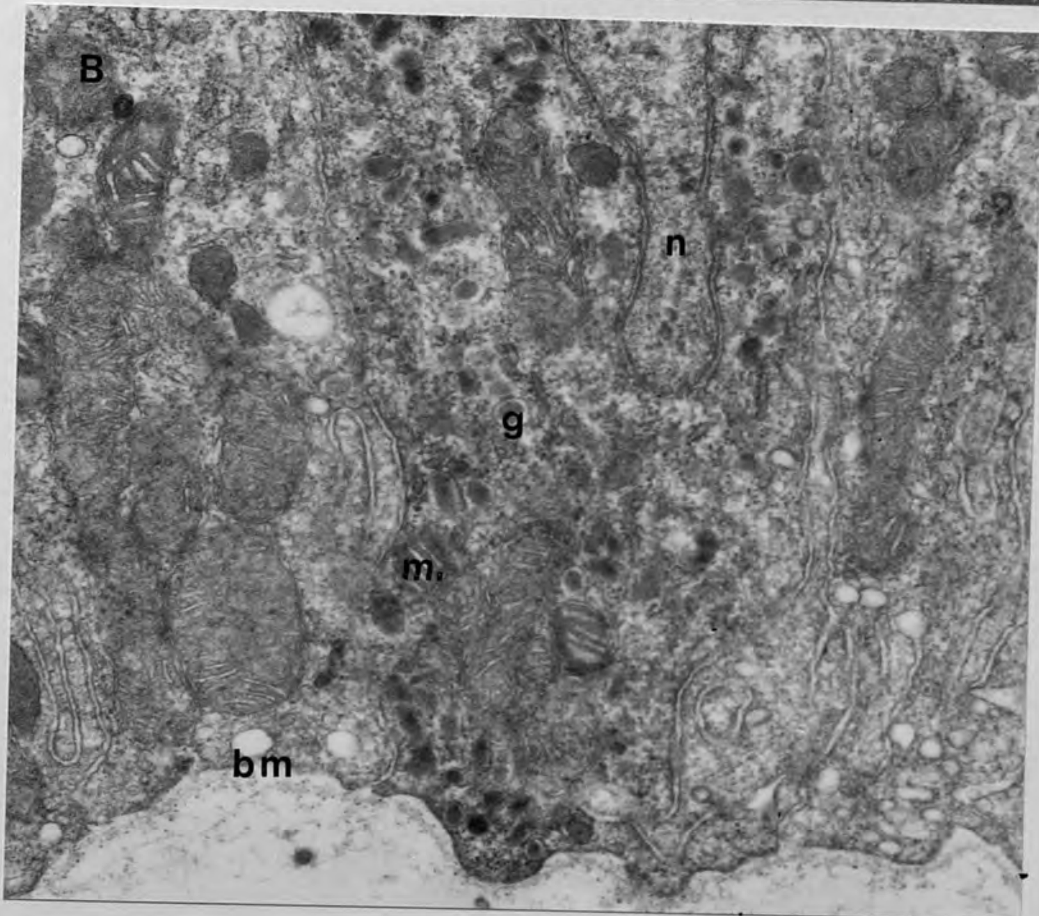
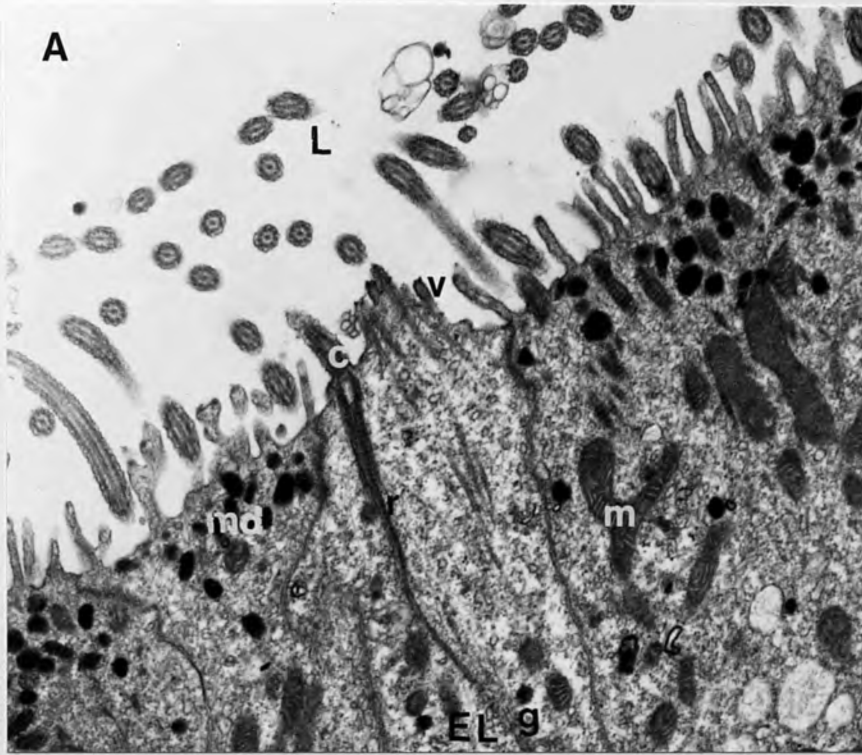
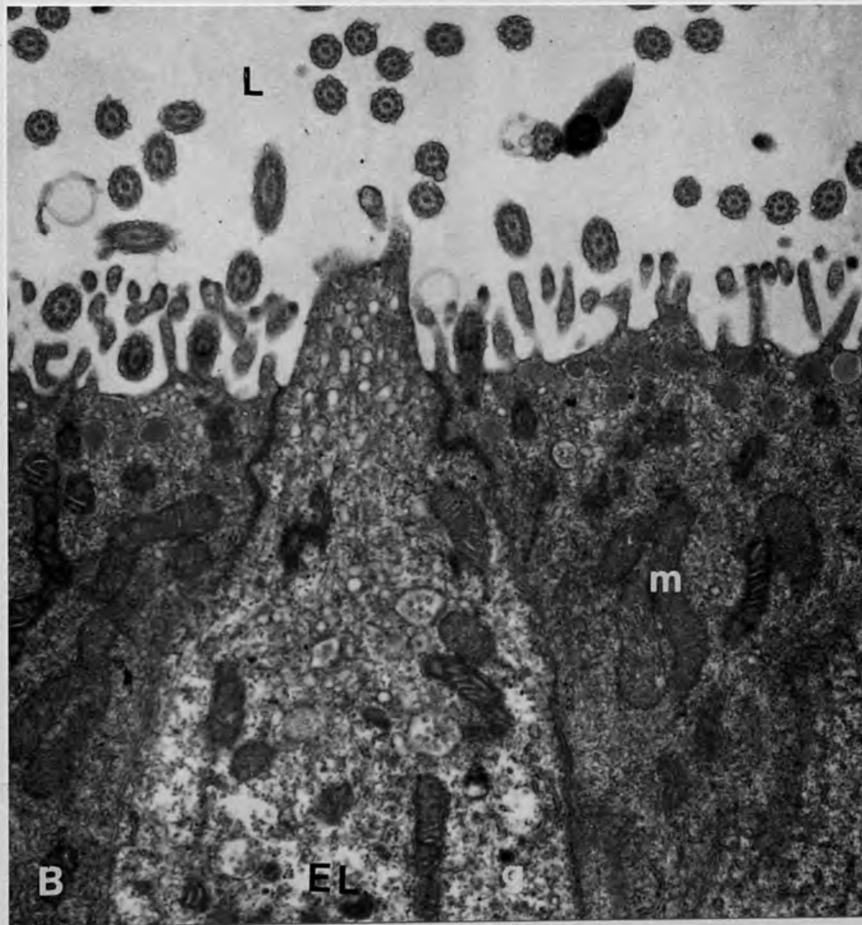


Fig 1:9 Medium power electron photomicrographs showing the apical regions of two E-L cells from the gastric mucous 'cap' region. In the E-L cells there are no mucous droplets, such as are packed into the apical regions of the surrounding mucous cells. The apices of the mucous cells contain large numbers of mitochondria, which are absent from the E-L cells. The apices of these E-L cells frequently protrude slightly into the gut lumen, and are tipped with a tuft of cilia and microvilli. A prominent ciliary rootlet can be seen in plate a. Almost no endocrine granules can be seen in this region of the cell.
c = cilia; EL = E-L cell; g = endocrine granule;
L = gastric lumen; md = mucous droplet; r = ciliary rootlet; v = microvilli. (a, x 14,250; b, x 17,500)

Fig 1.9





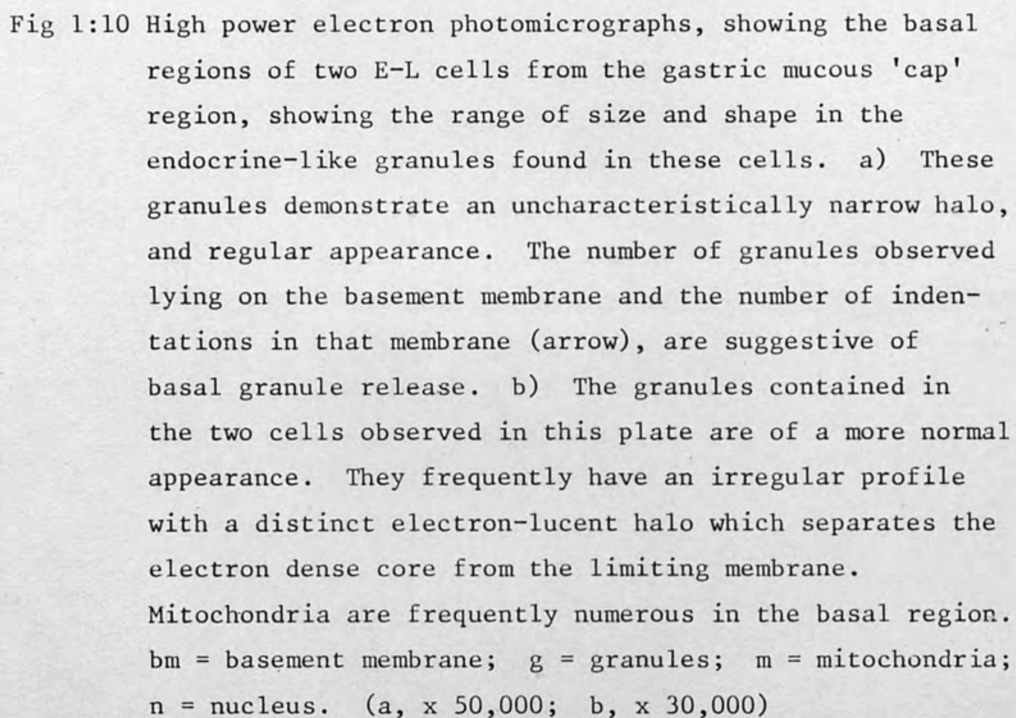
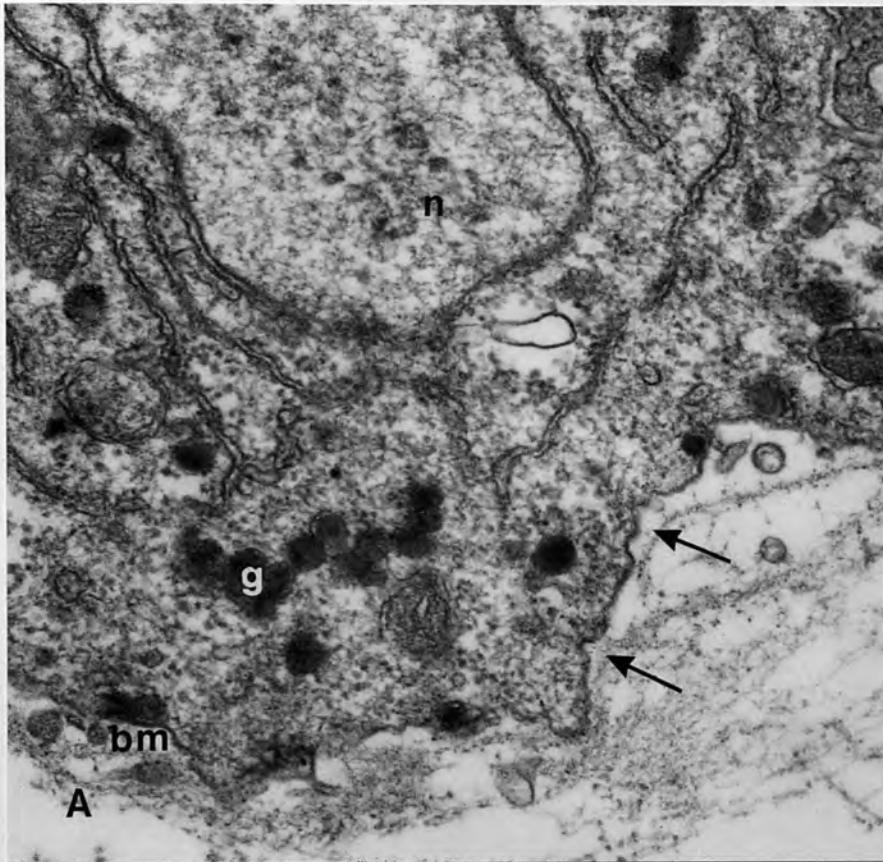
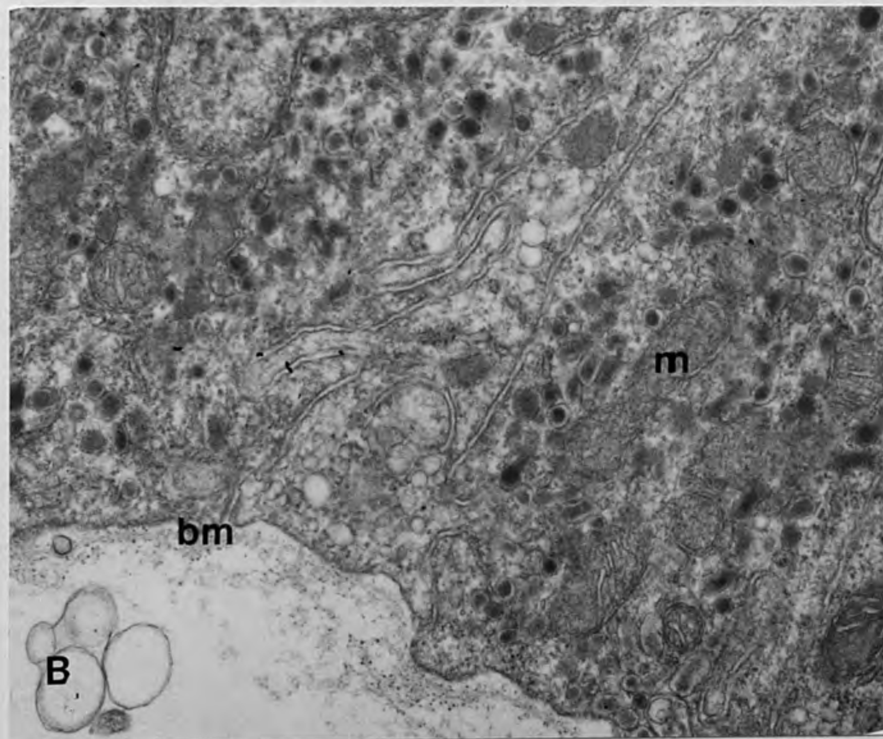


Fig 1:10 High power electron photomicrographs, showing the basal regions of two E-L cells from the gastric mucous 'cap' region, showing the range of size and shape in the endocrine-like granules found in these cells. a) These granules demonstrate an uncharacteristically narrow halo, and regular appearance. The number of granules observed lying on the basement membrane and the number of indentations in that membrane (arrow), are suggestive of basal granule release. b) The granules contained in the two cells observed in this plate are of a more normal appearance. They frequently have an irregular profile with a distinct electron-lucent halo which separates the electron dense core from the limiting membrane. Mitochondria are frequently numerous in the basal region. bm = basement membrane; g = granules; m = mitochondria; n = nucleus. (a, x 50,000; b, x 30,000)


n
g
bm
A


m
bm
B

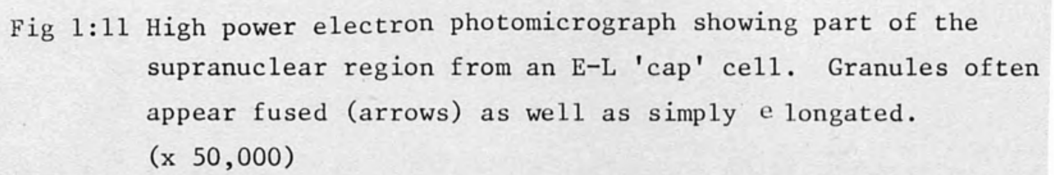
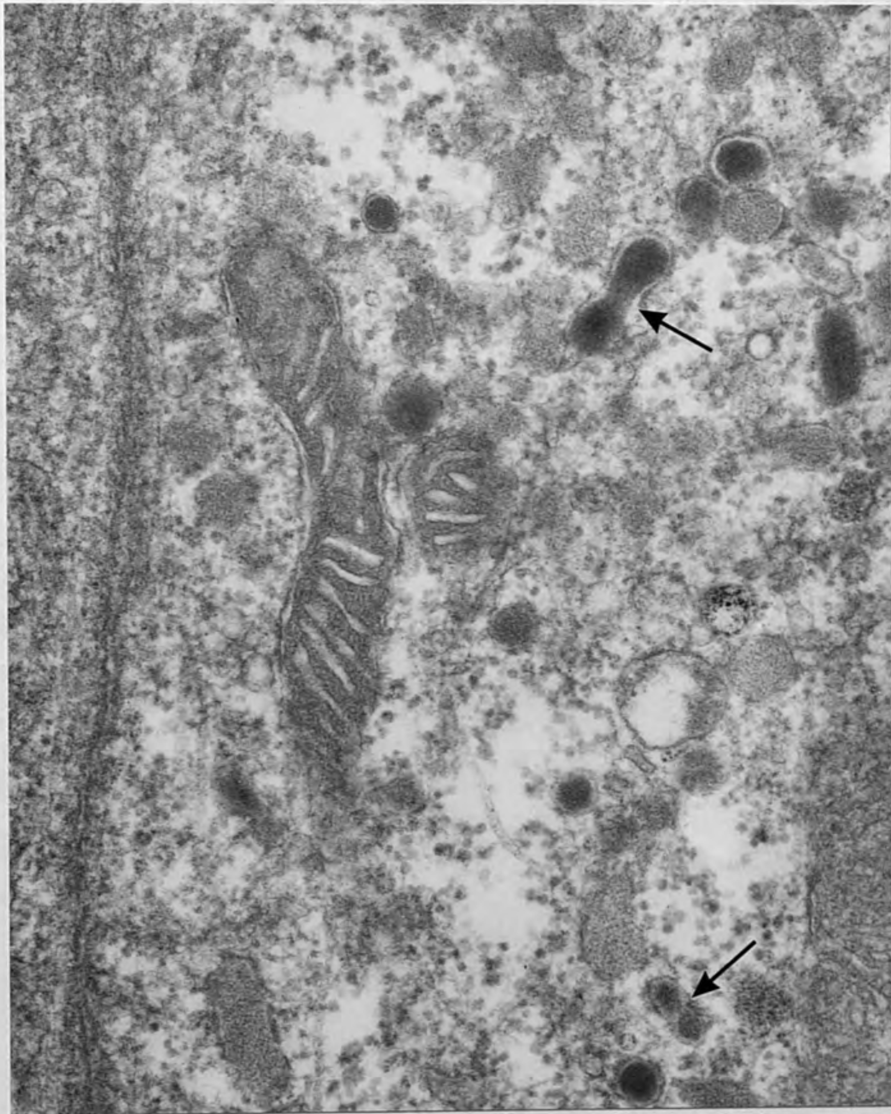


Fig 1:11 High power electron photomicrograph showing part of the supranuclear region from an E-L 'cap' cell. Granules often appear fused (arrows) as well as simply elongated. (x 50,000)



thinned out considerably towards the apex. Granules were often intimately associated with the basal membrane and occasionally characteristic omega profiles were observed (Fig 1:10), indicating basal release into the rete of blood capillary spaces which ramify below the epithelium.

The individual granules (Fig 1:8, 1:10) varied somewhat in appearance but had a frequently irregular profile with a normally distinct electron lucent halo separating the electron dense core from the limiting membrane. Granules occasionally appeared elongated or even fused (Fig 1:11) and were small, varying in size between 50 to 155 nm in diameter (90 nm average). Mitochondria were never particularly numerous and the Golgi apparatus was rarely well developed. The cytoplasm was filled with largely vesicular smooth endoplasmic reticulum, interspersed with free ribosomes.

Intestine

Preliminary surveys of the intestinal epithelium confirmed the histochemical observations of Section 1. Over much of the intestinal epithelium, E-L cells were absent.

SECTION 3 - IMMUNOCYTOCHEMISTRY

MATERIALS AND METHODS

Small pieces of gut from animals collected from Portsmouth were rapidly excised into cold sea water (4°C) and fixed in sea water Bouin's fluid (appendix 1) for 3 hrs at the same temperature. The tissue was then dehydrated in a graded series of ethanols, cleared in xylene and embedded, using a vacuum oven, in 56°C mp paraffin wax.

Sections were cut at 6 to 8 µ on a Jung rotary microtome and spread and mounted on albumenised glass slides at 37°C. Sections were then fully dried in a 37°C oven for 24 to 48 hrs before use.

Slides were subsequently stained either by the indirect fluorescent antibody technique (Coons et al, 1955) or the unlabelled antibody peroxidase-anti-peroxidase (PAP) method of Sternberger et al (1970).

Immunofluorescence

The paraffin sections were dewaxed in xylene which was then removed with petroleum ether. The slides were allowed to dry and were then spread out in a humid chamber. A 10 µl drop of the appropriate antiserum or control solution (Table 5) was then dispensed onto each section. This procedure made for economic use of antisera, as if wet slides were used, the solutions did not stay on the sections but spread out over the slides.

The slides were incubated for 18 hrs at room temperature after which the slides were washed in phosphate buffered saline (PBS) (appendix 1). The slides were then replaced in the humid containers and treated for 1 hr with FITC conjugated swine or goat anti-rabbit serum (Nordic), diluted 1:30 with PBS. Following a further wash in PBS the sections were mounted in buffered glycerine (9 parts glycerine to 1 part PBS) and examined with a Zeiss Universal Epi-fluorescence photomicroscope equipped with filters selected to give peak excitation at 490 nm (excitor, KP490, LP440; reflector, FT510; barrier, BP520-560).

Immunoperoxidase

Although the PAP technique was more time consuming than conventional fluorescence methods, it was considerably more sensitive. This had the effect of enabling more dilute antisera to be used, thus reducing any non-specific interactions between plasma and tissue. It also helped to conserve precious antisera. A second advantage of PAP staining was that, unlike fluorochrome labelling, it was permanent and did not fade during observation. Also it could be viewed with white light and sections counterstained, facilitating the observation of surrounding structures.

The paraffin sections were dewaxed in xylene and then transferred to absolute alcohol. The slides were immersed in fresh 0.03% hydrogen peroxide in methanol for 30 mins to block endogenous peroxidase activity. Following a rinse in 0.9% sodium chloride and then 15 mins in tris-HCl buffer (pH 7.2) the slides were immersed for 30 mins in the same buffer containing 0.5% crystalline bovine serum albumin and then replaced in the original tris-HCl buffer for 15 mins. The sections were then placed into an humidity chamber and treated with optimally diluted antisera or controls and left for 24 hrs at 4°C.

The slides were next washed in three washes of tris-HCl buffer (pH 7.2) and replaced in the humidity chamber. The sections were then treated at room temperature with unlabelled swine anti-rabbit IgG, diluted 1:20 with tris-HCl buffer. Following a wash with the buffer, the slides were replaced in the humidity chamber and the sections covered with rabbit peroxidase-anti-peroxidase complex (PAP) 1:50, at room temperature for 30 mins. The slides were washed through tris-HCl (pH 7.2) and then tris-HCl (pH 7.6).

The end product was then revealed with a freshly made solution of 5 mg 3,3' diaminobenzidine tetrahydrochloride dissolved in 10 ml 0.2 M

tris/HCL buffer (pH 7.6) to which 0.1 ml fresh 1% hydrogen peroxide had been added immediately prior to use. The depth of staining was controlled visually (10 to 20 mins). The slides could at this stage be counterstained with weak haematoxylin, cleared in xylene and mounted in DPX for permanent storage.

Antisera to a wide range of peptides were obtained from a number of sources, most being generously donated by Prof G Dockray (Liverpool) and Miss S Van Noorden and Prof J M Polak (RPGMS). These are listed in table 5. In addition to these gifts, an antiserum to secretin was prepared by the following method.

Preparation of antiserum

Antiserum was prepared after the method of Goodfriend et al, 1964. One mg porcine secretin (Sigma), 10 mg bovine serum albumin and 10 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride were separately dissolved in three 200 µl aliquots of 0.1 M phosphate buffer, pH 7.3. These were then pooled and gently agitated at room temperature for 2 hrs. The reaction was then terminated by dialysis against 400 ml of phosphate buffered saline (PBS) pH 7.4. The resultant conjugate was then added to 1 ml Freund's complete adjuvant (Difco) and thoroughly emulsified by repeated aspiration and explusion from a syringe fitted with a 21 g needle. The homogenate was then injected subcutaneously into an adult female New Zealand white rabbit, evenly divided between six sites about the neck and shoulders.

This procedure was repeated after six weeks and thereafter every three months. About 10 ml of blood was taken 10 days after each three-monthly booster, and stored overnight at 4°C. The clot which formed was gently removed and the plasma was stored in small ampouled aliquots at -20°C. For convenience this antiserum will be referred to

as secretin A .

Serum specificity

Two approaches were employed to assess the specificity of secretin A:

1) After determining the working dilution (by staining a series of sections with varying dilutions of antiserum), the diluted antiserum was incubated overnight with a range of concentrations of the parent antigen. This procedure demonstrated that incubation of the diluted antiserum (1:5000) with 10nmoles/ml secretin abolished staining.

2) The antiserum was incubated with either glucagon, VIP or CCK (50 nmoles/ml) under the same conditions described above; there was no effect on staining intensity.

Controls

Where positive immunostaining occurred, control staining was carried out using serum which had been exposed to the appropriate antigen (25nmoles/ml, at working dilution). This was carried out using antiserum diluted by the appropriate amount, alongside identical anstiserum which was to be used at the same time. This ensured that any factor other than an interaction between the antibody and antigen would affect both samples. They were left overnight in sealed ampoules at 4°C, and then used the following day. Control staining was also carried out using PBS or non-immune serum in place of the active serum. Positive controls were carried out by staining similarly processed mouse tissues.

TABLE 5

Antiserum	Antigen	Working dilution	Specificity	Source
L48	CCK-8, conjugated to BSA	1/1000	G-17 and CCK-8 equally	Prof G Dockray
L112	G-4, conjugated to thyroglobulin	1/1000	Gastrin/CCK COOH-terminal tetrapeptide	Prof G Dockray
L83	Substance P	1/1000		Prof G Dockray
L85	(VIP) porcine	1/1000	COOH-terminus	Prof G Dockray
L89	Bombesin	1/1000		Prof G Dockray
53	Secretin (natural)	1/2000	Some VIP X-Reactivity	RPGMS
278	Gastrin (synthetic human)	1/1000	COOH Terminal	RPGMS
38	Glucagon (natural)	1/1000	Some PP X-Reactivity	RPGMS
460	Somatostatin (synthetic)	1/1000		RPGMS
	CCK9-20	1/1000		RPGMS
	Insulin (bovine)	1/100		Wellcome
A	Secretin, conjugated to BSA	1/5000	No VIP, CCK or glucagon X-reactivity	Bedford

RPGMS = Royal Postgraduate Medical School

RESULTS

Oesophagus

Positive immunofluorescent staining was observed, using anti-insulin serum diluted 1:10 and 1:100. This immunofluorescence was not observed after pre-exposing diluted antiserum to 100nmoles/ml bovine insulin. Cells were observed throughout the oesophageal epithelium, in a basal position but extending by a slender process towards the gut lumen (Fig 1:1a). These were not observed with controls. These cells were not visualised using antisera to secretin (53), secretin (A), gastrin (278), glucagon (38), or somatostatin (460), even at dilutions considerably less than their normal working dilutions. All mouse control tissues were stained with the appropriate antisera.

Stomach

Sections of gastric epithelium were tested with both the immunofluorescence and PAP techniques, using all the antisera listed in table 5.

Positive results by both methods were observed with secretin (53) and secretin (A) (Figs 1:2a; 1:12; 1:13: 1:14). No immunoreactive cells could be visualised using any of the other antisera, although their activity could be demonstrated in mouse tissues.

The secretin immunoreactive cells occurred within the mucous 'caps' of the gastric epithelial ridges, in varying numbers. Very rarely a section of a cap was found that did not contain any immunoreactive cells, but normally caps contained between one and five, and occasionally even more. This immunoreactivity could be abolished by the pre-exposure of the diluted antiserum to 10nmoles/ml secretin and both

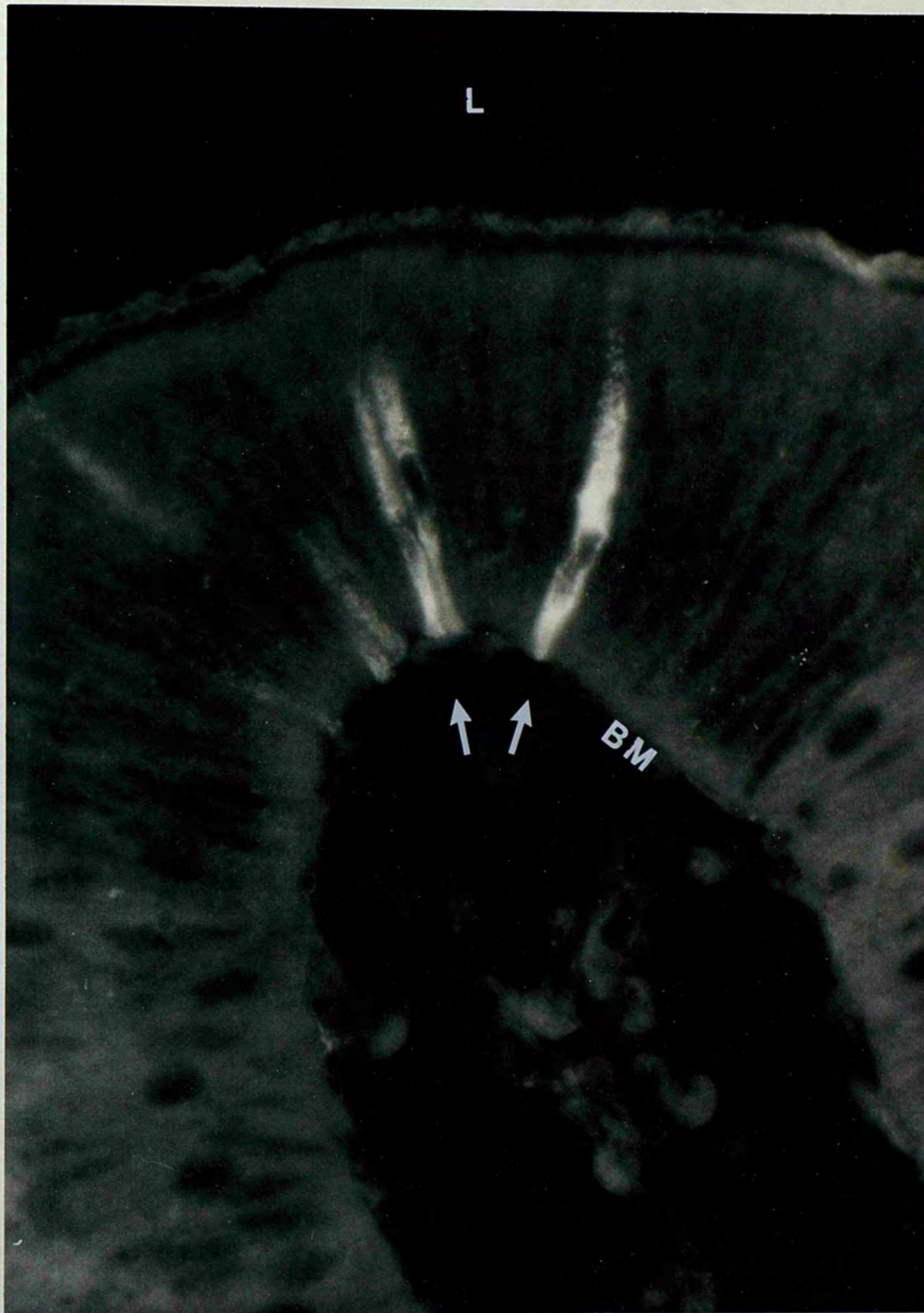


Fig 1:12 Light photomicrograph of secretin-immunofluorescent cells (arrow) in the gastric 'cap' region. Anti-secretin serum 53, diluted 1:5000, x 750. L = lumen; BM = basement membrane.

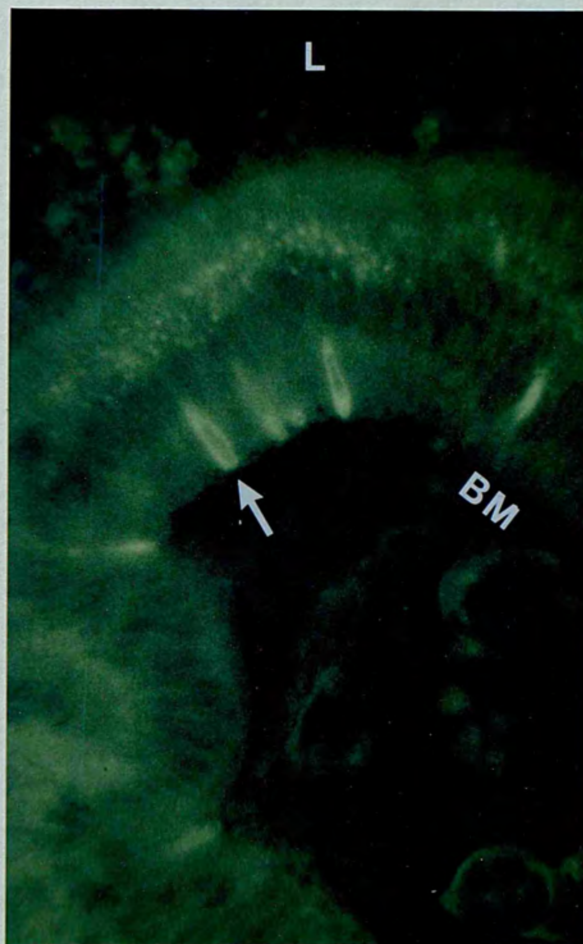


Fig 1:13 Light photomicrograph of the secretin-immunofluorescent cells (arrow) in the gastric 'cap' region. Anti-secretin serum A, diluted 1:5000, x500 . L = lumen; BM = basement membrane.

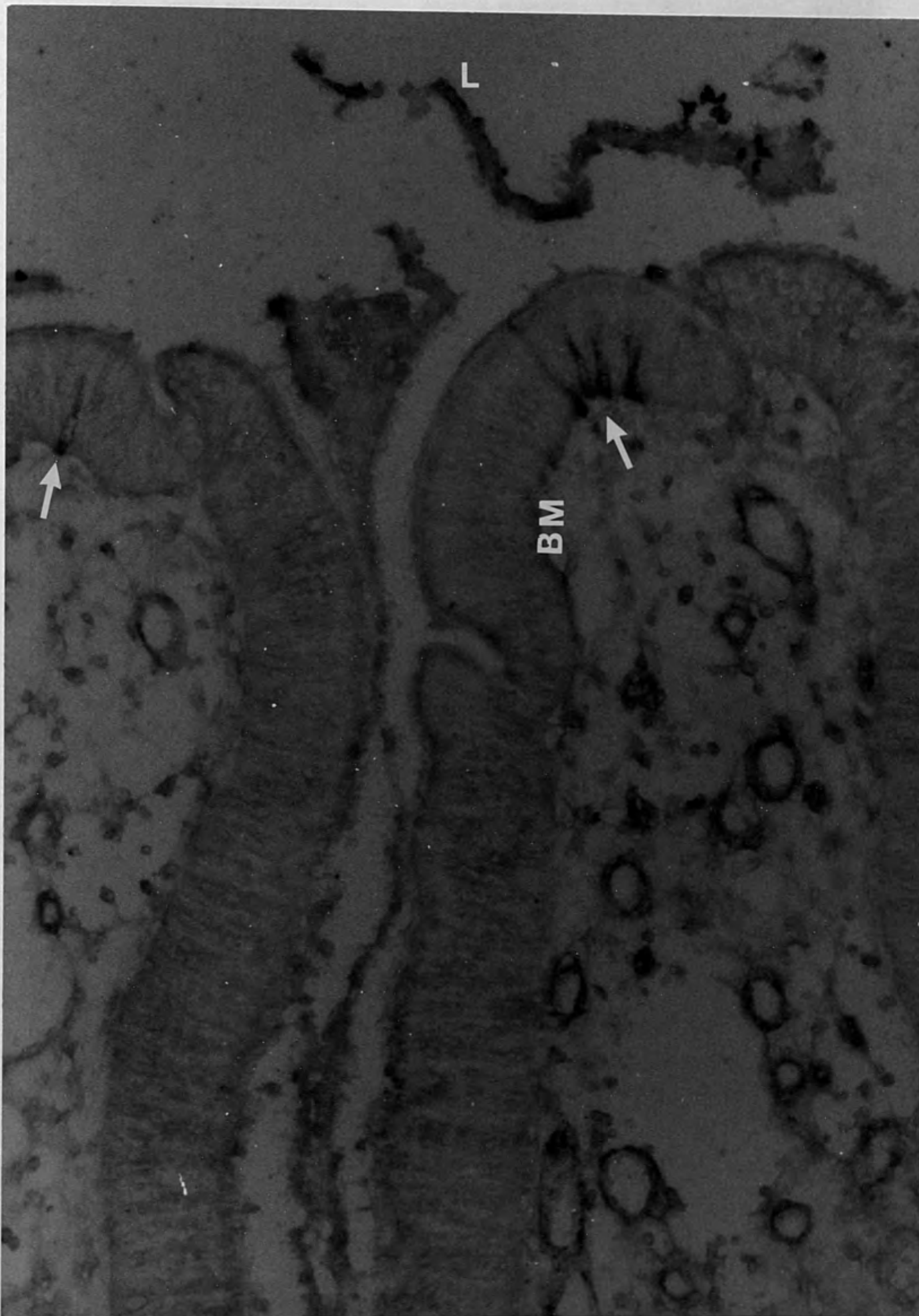


Fig 1:14 Light photomicrograph of secretin-immunoreactive cells (arrow) in the gastric 'cap' region. Anti-secretin serum 53, diluted 1:9000, PAP method, x 350. L = lumen; BM = basement membrane.

Fig 1.15

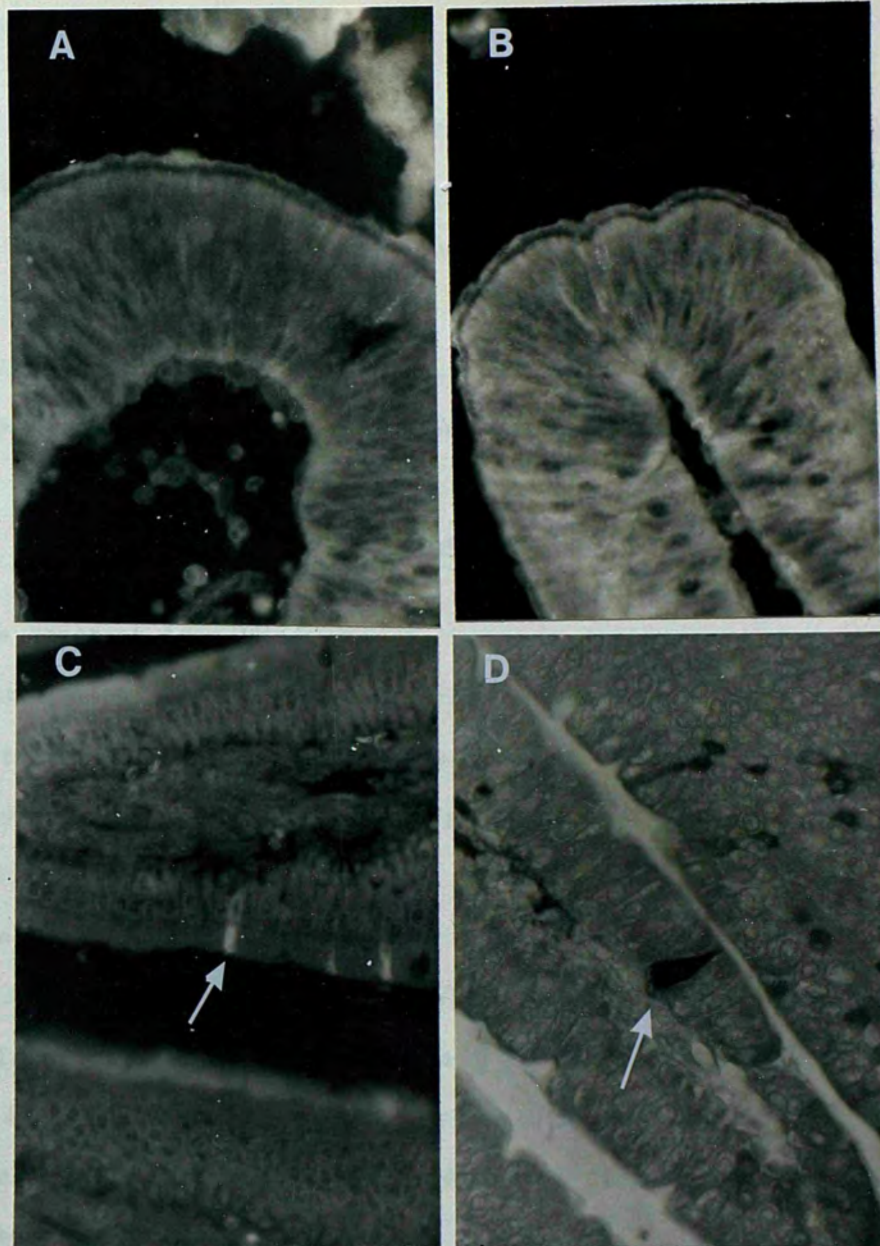


Fig 1:15 a) Immunofluorescence of Styela gastric 'cap' after incubation with anti-secretin serum 53, (1:5000) preabsorbed overnight with 10 nM/ml secretin x 250. b) Immunofluorescence of Styela gastric cap after incubation with anti-secretin serum A, (1:5000) preabsorbed overnight with 10 nM/ml secretin x 250. In both a) and b) staining of E-L cells has been abolished. c) Immunofluorescent localisation of secretin in immunoreactive cell (arrow) in mouse duodenum, incubated with anti-secretin serum A, x 250. d) PAP localisation of a secretin immunoreactive cell (arrow) in mouse duodenum using anti-secretin serum 53, x 250.

secretin antisera could be used to demonstrate immunoreactive cells in mouse duodenum (Fig 1:15).

The secretin-positive immunoreactive cells visualised by both methods showed an identical appearance and distribution. They lay on the basal lamina of the 'cap' epithelium and extended towards the gut lumen by a typically slender tapering process. The immunoreactivity appeared throughout the cells, but the most intense staining occurred basally, particularly about the prominent nucleus.

DISCUSSION

It is apparent from the results that the basal granulated cells in the gut of Styela bear some resemblance to the hormone producing cells of the vertebrate gastro-entero-pancreatic endocrine system (for reviews cf Falkmer and Ostberg, 1977; Fujita and Kobayashi, 1977; Pearse, 1974; Solcia et al, 1978b). The general ultrastructural appearance of these cells in Styela is similar to that of APUD cells (Pearse, 1968a, 1969). Like APUD cells they contain little rough endoplasmic reticulum, but they do contain smooth endoplasmic reticulum in the form of vesicles. Free ribosomes and electron dense mitochrondria are prominent, but most distinctive and typical are the membrane bound secretion vesicles.

The appearance of these secretion vesicles has been used as the basis of a classification of endocrine cell types at the Symposium on Gastrointestinal Hormones, held in Weisbaden in October 1969 (Creutzfeldt et al, 1970). Despite being updated (Solcia et al, 1973) and completely revised (Solcia et al, 1978) these classificatory systems are of little direct use in determining the nature of the E-L cells of Styela, as they were specifically developed for use with

mammalian and in particular, human, gastro-entero-pancreatic endocrine cells.

There are, however, clear differences between the granule morphology of the gastric and oesophageal E-L cells of Styela. Whereas the gastric granules are small (50 to 155 nm, 90 nm av), irregular in shape and electron density and have a distinct 'halo', the oesophageal granules are larger (100-175 nm, 130 nm av), more regular in shape and electron density, and have a narrow or non-existent 'halo'. No other attempt has been made to distinguish between E-L cell types in ascidians from ultrastructural evidence.

E-L cell types have been described from the gut of a number of ascidian species. One endocrine cell type has been observed in the gastric epithelium of Botryllus schlosseri (Burighel and Milanesi, 1975), Halocynthia papillosa (Pestarino et al, 1982), Styela plicata (Pestarino, 1982a; 1982b) and Ciona intestinalis (Fritsch and Sprang, 1977), although in this last example, there is considerable histological and immunofluorescence evidence that more than one type of E-L cell is present (Fritsch, 1976; Fritsch et al, 1978).

Welsch and Dilly (1980) have been unable to demonstrate any E-L cells in the hemichordate gut epithelium, but work on cyclostomes, the most primitive vertebrates, has demonstrated a single gut endocrine cell type (Ostberg et al, 1976; Van Noorden and Pearse, 1974).

Observations on the digestive epithelium of Branchiostoma (Kataoka and Fujita 1974; Van Noorden and Pearse, 1976; Welsch, 1975) have distinguished at least two cell types and possibly three or four, on the basis of their granule morphology. Thus in Branchiostoma, type I cells have dense, rather irregular granules with an obvious 'halo' and an average size of 140 nm. Type III cells are similar but smaller

(average diameter 60 nm) and with the granules eccentrically placed within their surrounding membranes. The granules of the gastric and intestinal E-L cells of Styela have a similar appearance to the type I and type III cells of Branchiostoma, and a size range that lies somewhere between the two (50 to 155 nm, 90 nm average). These cells will be referred to as Styela type I cells.

The Branchiostoma type II cells show close similarities to the Styela oesophageal cells, which will therefore also be referred to as type II cells. Like them, the Branchiostoma type II cells have a more rounded appearance, a narrow or non-existent halo, and an average diameter of 170 nm. They also, like Styela type II cells, stain with aldehyde fuchsin and immunoreact with anti-insulin serum (Van Noorden and Pearse, 1976).

The E-L cells described by Fritsch and Sprang (1977) in Ciona are similar to Styela type I, although the granules are somewhat larger. However when attempting to compare granule size and appearance it must be born in mind that these factors can be markedly affected by the method of fixation employed (Mortensen and Morris, 1977). It is therefore probably safe to say that in Styela where the same fixation routine has been followed throughout, there are two distinct populations of granulated cells. The comparison of these with superficially similar cells in Branchiostoma and Ciona is perhaps unwise. A clearer insight into the nature of the product of these cells is provided by the histochemical and immunocytochemical staining reactions which have been demonstrated.

The gastric type I cells of Styela exhibit a formaldehyde induced fluorescence (FIF), indicating that they contain a biogenic monoamine (Fig 1:2b). Similar cells have been recorded in the gastric epithelium

of Ciona (Erspamer,1946), and in the gut epithelium of a large number of other ascidians (Gerzeli, 1964). The mechanism by which certain biogenic monoamines are converted to fluorescent derivatives is well established (Falck et al, 1962; Fuxe and Jonsson, 1973). Initially they react to form a weakly fluorescent intermediate, which is subsequently dehydrogenated and incidentally made insoluble to form an intensely fluorescent dihydro derivative. This method displays a considerable sensitivity and as little as 0.5 ng of noradrenaline can be detected within a single nerve terminal (Fuxe and Jonsson, 1973). No attempt was made to quantitate the amine content, but the level of fluorescence obtained, compared to mouse adrenal gland, was low.

The argentaffin staining reaction is dependent on the reduction of silver salts by a tissue component such as a biogenic monoamine. Therefore it is possible that the levels of amines located in the gastric type I cells are insufficient to cause this reduction. However, as it was not possible to stain these cells by argyrophil methods, where an exogenous reducing agent is added it must be concluded that these cells are not capable of accumulating silver. This is slightly surprising as argyrophil and argentaffin cells have been noted in the gastric epithelium of Ciona (Fritsch, 1976). Perhaps of more significance is the observation by Pestarino (1979; 1982b) that there are columnar cells within the mucous caps of Styela plicata that stain with phosphotungstic acid-haematein (Cavellero et al, 1968). But he finds that these cells do not stain with the Grimelius argyrophil method, and are non-argentaffin. Like the E-L cap cells of S. clava, those of S. plicata are not stained by aldehyde fuchsin or masked metachromasia. Three other supposedly endocrine cell types that Pestarino (1979; 1982c) located in the side walls of the gastric mucosa have no counterparts in S. clava and until the gastric epithelium of S.

plicata has been fully observed at an ultrastructural level it will be difficult to interpret this finding.

Another unexpected observation is that in Styela, type I cells stain with lead haematoxylin, though not masked metachromasia. These stains are both believed to act on the side-chain carboxyl or carboxyamido groups of granule proteins (Solcia et al, 1968; Solcia et al, 1969a). However both of these methods are empirical and neither are fully understood. It is not therefore possible to determine whether this result has any significance. Pestarino (1982b) finds that neither of these stains are taken up by secretin-like immunofluorescent E-L cap cells in Styela plicata. This finding is in partial agreement with the situation in Styela clava where the lead haematoxylin staining is only weak and the sections need to be exposed for longer than control tissues.

The oesophageal type II cells are also unstainable with masked metachromasia but they do stain with aldehyde fuchsin. A number of vertebrate structures are stained by aldehyde fuchsin, including elastic tissue, β-cells of the pancreas, pituitary β-granules, mast cell granules, mucins and gastric chief cells. Ortman et al (1966) have shown in the frog pituitary, that the staining is caused by a combination of the dye with carboxyl groups. In the staining of pancreatic β-cell granules, Bussolati and Bassa (1974) have suggested that the dye forms an association with sulphate groups caused by oxidation of disulphide bonds in the insulin molecule. However, although aldehyde fuchsin is not a specific staining method, taken in conjunction with the observation that these cells also display an insulin-like immunofluorescence, it would seem more likely that it is this second mechanism which is causing these type II cells to stain.

It is not completely certain that the immunofluorescent cells and the aldehyde fuchsin cells are identical, as although their appearance and position on the basement membrane are the same, the insulin-immunofluorescent cells are observed less frequently. Argyrophilic cells were observed only rarely and it is possible that the argyrophilic cells represent a second oesophageal cell type which was overlooked at the ultrastructural level. The reason that the immunofluorescent cells were observed less frequently than aldehyde fuchsin stained cells is possibly linked with the poor cross-reactivity of the cells with the antiserum. The cells which were observed may represent cells containing a full complement of granules, while other cells in a different phase of the secretory cycle, and hence partially degranulated, would show insufficient fluorescence to permit their observation. Immunofluorescence is a highly specific technique and it might be expected that protochordate peptides would be immunologically dissimilar to their mammalian counterparts. It is possible that an anti-avian insulin, if available, would display a higher affinity for 'ascidian insulin', as such a factor extracted from the gut of Ciona intestinalis, has been found to be antigenically closer to chicken insulin than mammalian insulins (Falkmer and Wilson, 1967).

These type II cells show many parallels with the type II cells in Branchiostoma (Van Noorden and Pearse, 1976), which can also be demonstrated by immunofluorescence using anti-human insulin. They also noted that positive immunofluorescence could only be obtained using certain antisera and only then by using higher concentrations than those used in vertebrate control tissues and by staining for an extended period of time. They also noted considerable variations between the individual animals and the degree of staining achieved for no discernable reason. Indeed with some batches of animals, no insulin

immunoreactive cells could be demonstrated (Van Noorden, personal communication). These insulin immunoreactive cells, when they could be visualised, restained with aldehyde-fuchsin, but they noted that other cells, which had not shown any immunofluorescence, were also stained. The ultrastructural appearance of the granules is also similar, as has already been noted.

Similar aldehyde fuchsin positive cells have been observed in the upper digestive tract of *Ciona intestinalis* by Falkmer (1969). These cells have not been tested with anti-insulin sera, but insulin activity has been achieved using an 'immunological bioassay' in the gut of *Ciona* (Davidson et al, 1971; Falkmer, 1969; Falkmer and Wilson, 1967).

The gastric type I cells readily immunoreact with anti-secretin sera but not with any of the other antisera listed in table 5. No E-L cells were observed by any of the methods employed anywhere in the gastric epithelium other than in the mucous 'caps'.

Since this work was carried out, the presence of secretin-like immunoreactivity has been confirmed by Pestarino and his co-workers in two ascidian species, *Halocynthia papillosa* and *Styela plicata* (Pestarino et al, 1982; Pestarino, 1982a; 1982b).

Styela plicata, like *Styela clava*, has a folded gastric epithelium topped by mucous caps which contain E-L cells which immunoreact with anti-porcine secretin, even after pre-incubation with VIP, GIP or glucagon. No immunofluorescent E-L cells were observed anywhere else in the gastric epithelium, although Pestarino does also describe anti-secretin immunoreactive cells in the oesophageal and intestinal epithelium (Pestarino, 1982b) which have not been observed in *Styela clava*.

The 'caps' are absent from the gastric epithelium of *Ciona intestinalis* (Yonge, 1925; Thomas, 1970) and in contrast to the situation in *Styela clava* and *S. plicata*, E-L cells appear scattered throughout the columnar epithelium of the gut (Fritsch, 1976). These cells demonstrate somatostatin-, gastrin- and neurotensin-like immunoreactivity (Fritsch et al, 1978; Reinecke et al, 1980). A CCK-8-like peptide has also been observed by radioimmunoassay in the gut of *Ciona* (Falkmer et al, 1981).

Secretin is phylogenetically old. It has been demonstrated in nerve fibres in the brain of the hoverfly larva, *Eristalis aeneus*, though not in the digestive tract (El-Salhy et al, 1980). It has also been localised in nerve fibres in the gut wall of a mollusc, the giant African land snail, *Achatina fulica* (Van Noorden and Falkmer, 1980; Van Noorden et al, 1980:). Secretin-like bioactivity has also been reported in extracts of the spiral caecum of the mollusc *Octopus vulgaris* (Ledrut and Ungar, 1937; Mutt, personal communication).

Van Noorden and Pearse (1974; 1976) have been unable to demonstrate a gastrointestinal cellular site of secretin production in either *Branchiostoma* or the lamprey *Lampetra fluviatilis*, although secretin-like activity has been demonstrated in intestinal extracts of various species of lamprey using bioassay (Barrington and Dockray, 1970; Nilsson, 1973). Attempts to immunostain secretin-producing cells in the gut of fish, amphibia and birds using mammalian anti-secretin have proved unsuccessful (Van Noorden and Falkmer, 1980) although secretin immunoreactive cells have been successfully demonstrated in reptilian gut by Buchan (1981).

Although the *Styela* 'cap' cells immunoreact with two secretin antisera, it is not known to which portion of the secretin molecule

these antisera are directed; only that control mammalian tissues also immunoreact. It is possible that the 'cap' cells contain an unrelated molecule which nevertheless shares the peptide sequence towards which these antisera are directed. To investigate this possibility it has been necessary to prepare extracts of *Styela* gut and test whether they have a secretin-like action in vertebrate systems.

CHAPTER 2 - ACTION OF EXTRACTS OF THE GUT OF STYELA CLAVA IN A RAT AND TURKEY BIOASSAY

INTRODUCTION

The secretin bioassay

The extraction of secretin

MATERIALS AND METHODS

Gut extracts
- a) Source of standard
- b) Source of tissues
- c) Processing of tissues

Operative procedure
- a) Rats
- b) Turkeys

Administration of test materials

RESULTS

The rat

The turkey

DISCUSSION

Possible causes of loss of activity
- a) Enzyme inactivation
- b) Interface inactivation
- c) Alginic acid

CONCLUSIONS

INTRODUCTION

The secretin bioassay

Any bioassay system relies on the quantification of a specific response to a specific challenge. In the bioassay of secretin, the responses which have been measured have been the production of water (Ivy et al, 1930) and bicarbonate ions (Hammarsten et al, 1928) by the exocrine pancreas.

The initial finding of secretin was in the dog (Bayliss and Starling, 1902a; 1902b) and many bioassay systems have subsequently made use of this animal (Ivy et al, 1930; Greengard and Ivy, 1938; Ivy and Janecek, 1959; Lin and Alphin, 1962; Vagne et al, 1968; Spingola and Grossman, 1973). The particular advantages of dogs are their sensitivity to secretin, and the reproducibility of results in any given dog (Lin and Alphin, 1962). They are, however, expensive to purchase and maintain, and require elaborate housing and trained personnel.

Cats have long been used as a cheaper and more easily maintained alternative (Hammarsten et al, 1928; Mellanby, 1928; Wilander and Agren, 1932; Burn and Holton, 1948; Ivy and Janecek, 1959; Mutt and Soderberg, 1959; Jorpes and Mutt, 1966; Konturek, 1969). They also have the advantage that they can be maintained, under anaesthesia, for two to five consecutive days by using sterile operating conditions and administering antibiotic therapy, and oral Placydil (ethchlorvynol) (Mutt and Soderberg, 1959). This considerably reduces the number of animals required.

However as facilities to maintain dogs and cats were not available, and a Home Office certificate E is required for operative

procedures on these animals, it was decided that neither species were suitable in this instance.

Rabbits have been used for the assay of secretin (Dorchester and Haist, (1952), though they have not been used by other workers. They are reported to readily succumb to operational trauma (Mutt and Soderberg, 1959) and display a high level of spontaneous pancreatic secretion (Jorpes and Mutt, 1973).

A technique using turkeys (Dockray, 1972b; 1975a) has proved more sensitive than has a rat bioassay, to intestinal extracts of pike, *Esox lucius* (Dockray, 1974) and cod, *Gadus morhua* (Dockray, 1975b). This effect is presumably due to structural differences between fish and mammalian secretins. Although fish secretins have not been characterised, chicken secretin has about 50% sequence dissimilarity to porcine secretin (Fig 3:9) (Carlquist and Mutt, 1980) and this dissimilarity might be expected to be even greater for *Styela* 'secretin'. Thus from this point of view a turkey bioassay was considered to be a desirable choice. There were however problems associated with accommodating numbers of birds between their purchase and use, and for this reason it was decided that in the first instance, a rat bioassay should be tested to determine its ability to recognise *Styela* 'secretin'.

The assay of secretin using rats was pioneered indepedently by Love (1957) and Svatos and Jelinek (1957). They have since been commonly used (Debray et al, 1962; Lin and Alphin, 1962; Heatley, 1968a; 1968b; Tachibana, 1971; 1973; Dockray, 1972a; Schmidt et al, 1972), probably because of the low cost of their purchase and maintenance, and the ready availability of standardised laboratory strains.

However this bioassay suffers from two disadvantages. Firstly, the rat appears to be insensitive to secretin, compared with other species which have been used (Dockray, 1972a). Lin and Alphin (1962) found dogs to be forty times more sensitive to secretin than were rats, calculated on the basis of dose per kilogram body weight. This apparently major problem is not however serious, due to the considerably smaller body weight of the rat, which therefore responds over much the same range of doses as does the dog.

The second complication arises because, in the rat, as well as stimulating pancreatic enzyme secretion (ecbolic effect), CCK also promotes the flow of pancreatic fluid (hydrelatic action) (Heatley, 1968a; Dockray, 1972a). Two solutions to this problem have been proposed by Heatley (1968a). Both secretin and CCK may be assayed simultaneously, and then a correction can be made for the calculated hydrelatic action of the CCK present in the test material. Alternatively the CCK activity of the sample can be selectively destroyed using the hydrogen peroxide method of Mutt (1964).

In the rat, pure CCK has a hydrelatic action, with a potency approaching one quarter of that of pure secretin (Heatley, 1968a). Thus a sample containing CCK but no secretin, could, if assayed without using one of the two above mentioned controls, be mistaken for a sample containing secretin.

Thus it would be imperative to use one of these variations in a bioassay of Styela gut extract to ensure that the hydrelatic response was not entirely due to a CCK-like molecule, present in the gut, which had not been detected by immunocytochemical means.

These considerations would only apply however if a hydrelatic response to the extracts was obtained, and as this was far from certain

it was decided to conduct a pilot study in the first instance. This would be used to test: a) commercial secretin, to test that the bioassay was working correctly; b) extracted pig duodenum, to test that the extraction method was working correctly; c) Styela gut extract, to test whether it contained any material which provoked a hydrelatic response, and d) extracts of control tissues (known not to contain secretin), to ensure that the hydrelatic action was not an artefact produced by some other unknown factor.

If a), b) and c) produced a hydrelatic effect, and d) did not it would then be necessary to determine to what extent the effect in c) was caused by CCK-like factors.

The extraction of secretin

In 1902, Bayliss and Starling reported that dilute aqueous hydrochloric acid extracts of the canine jejunal mucosa contained a substance which caused pancreatic secretion when injected into the bloodstream of dogs (Bayliss and Starling, 1902a, 1902b). This substance they named 'secretin'.

They found that it was thermostable and non-volatile, insoluble in ethanol and ether, though soluble in aqueous ethanol; it dialysed through parchment, and was inactivated by dilute aqueous potassium permanganate, proteolytic enzymes and the salts of mercury, lead and iron.

Their secretin extracts were prepared by boiling the upper intestinal mucosa in 0.4% hydrochloric acid. This was then neutralised with sodium hydroxide, while still boiling, and allowed to cool. The mixture was then re-acidified with acetic acid and filtered. Ethanol was then added to the filtrate, which caused the precipitation of many

impurities, while the secretin remained in solution.

The purification techniques have been the subject of a number of excellent review papers (Still, 1931; Greengard, 1948; Grossman, 1950; Mutt, 1959b) and will therefore not be fully discussed here. However some of the more important stages in the development of the purification techniques will be mentioned insofar as they bear on the choice of a method for preparing Styela extracts.

All attempts to obtain secretin in a chemically pure form were unsuccessful until recently (Jorpes and Mutt, 1961) because the necessary separation techniques, such as column chromatography and counter-current distribution had not been developed. However in the half century which preceeded its eventual isolation a considerable amount of information regarding its properties was derived.

No significant advances on the original preparations of Bayliss and Starling (1902a; 1902b) were made until the mid 1920s, when researchers began to change from canine to porcine material, which could be obtained in far greater quantities. Thus sufficient material could be produced to enable them to undertake more extensive purification than had hitherto been possible. This was coupled with an important development, separately introduced by Penau and Simonnet (1925) in France, and Weaver et al (1926) in America, which involved the separation of secretin from toxic, low molecular weight impurities, by salting it out from aqueous solutions with neutral salts.

Another important development, pioneered during this era, was the adsorption of secretin, which was known to be basic, onto a weak, insoluble acid, followed by its subsequent elution by a stronger acid. Thus by careful choice of these acids, materials with a lower pH than secretin would not be adsorbed, and materials with a higher pH would

not subsequently be eluted. This enabled a considerable factor of purification to be made at an early stage in the extraction process.

The choice by Mellanby (1928) of precipitated bile acids as an adsorbant was perhaps unfortunate, as the variability of commerical bile salt preparations rendered his successful purification procedure unrepeatable when attempted by others (Mortimer and Ivy, 1929; Still, 1931). This approach was therefore not further pursued until it was refined by Jorpes and Mutt during the 1950s.

Using a modification of the method of Friedman and Thomas (1950), Jorpes and Mutt (1952) originally made secretin preparations by extracting pig intestines with dilute aqueous hydrochloric acid from which, after filtering, the secretin could be salted out by the addition of sodium chloride.

They encountered problems however, in filtering these extracts, and tried to circumvent this step by extracting with acid alcohol, and subsequently distilling off the liquid, rather than filtering. Unfortunately the residues were rich in lipids and equally difficult to handle.

This led them to seek a water and alcohol insoluble material upon which to adsorb the secretin from this initial extract, so that the troublesome, lipid rich acid alcohol could be filtered off and discarded. Their subsequent experiments with ion exchange resins and pectic acid have been comprehensively reviewed by Mutt (1959b) but none of the materials which they used were entirely satisfactory.

Alginic acid, when it was tried, appeared to have considerable advantages. However it would only adsorb secretin from aqueous solutions. To reduce the original problems of working with aqueous

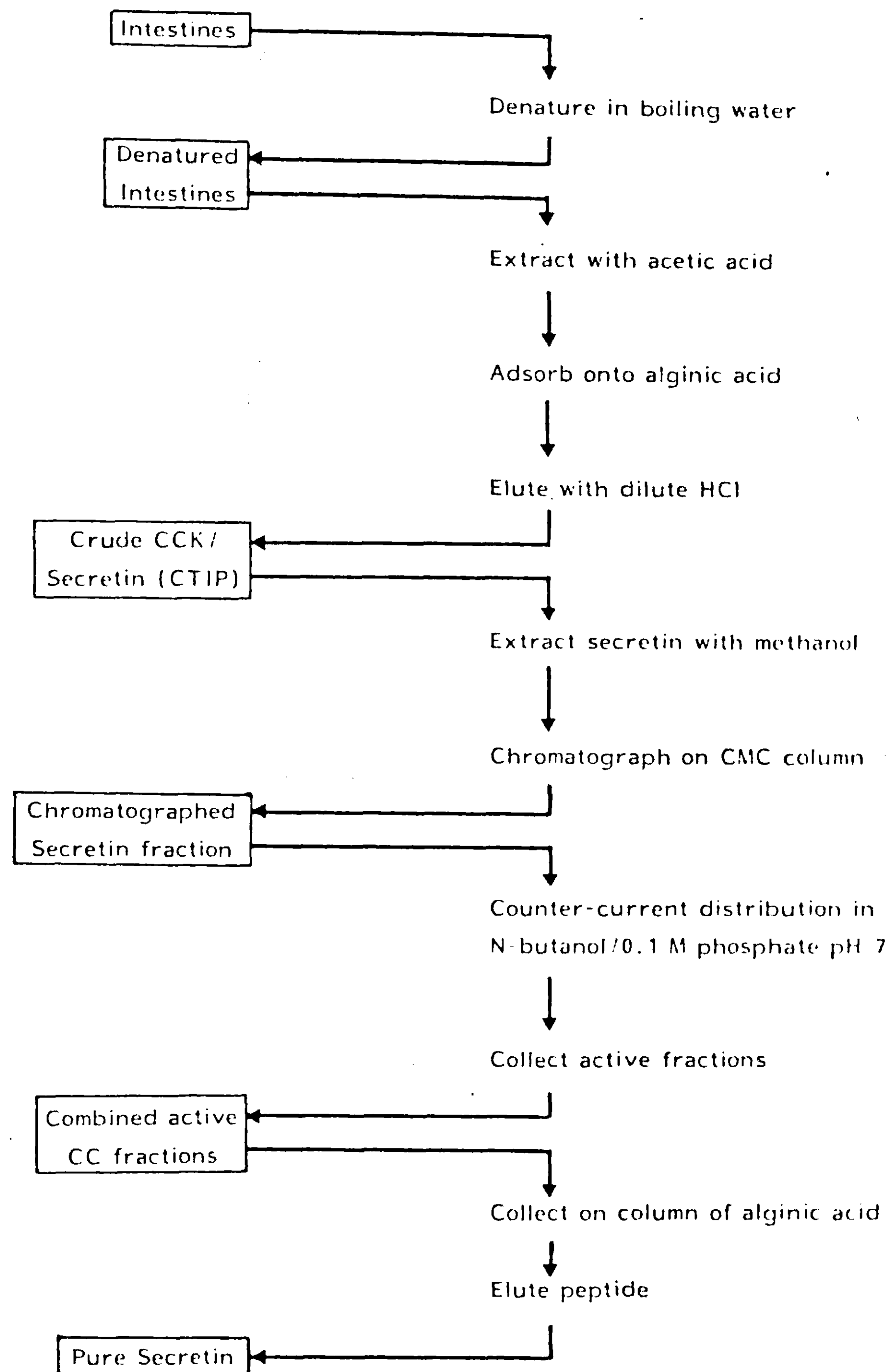


Fig 2:1 Flow diagram showing the original scheme for the production of biologically pure secretin. After Jorpes and Mutt, 1967.

extracts they boiled the intestines for a few minutes in water, to destroy the proteolytic enzymes and stabilise the secretin. Subsequent extraction at room temperature with dilute acetic acid left most of the coagulated proteinaceous material and lipids undissolved in the intestine, which made the extracts much easier to filter, greatly facilitating the removal of the intestinal tissue

Alginic acid was then added to the filtrate, to adsorb the secretin, which was subsequently eluted with dilute hydrochloric acid. The secretin could then be precipitated from the eluate with sodium chloride (Mutt 1959a).

This concentrate of thermostable intestinal peptides (CTIP) could then be processed further to purify the secretin (Fig 2:1) or indeed any of the many other peptides which it contains (Mutt, 1976; 1978; 1980).

It was found at an early stage in the present study that the amount of CTIP that could be prepared from Styela stomachs was extremely small, compared to that produced from a comparable weight of pig gut. Also that the stomachs of approximately 1,000 Styela weighed the same as the first metre of duodenum from one pig (300 g). Thus the amounts that could reasonably be prepared did not allow for any further purification of the crude CTIP.

It was therefore decided that Styela stomach CTIP and pig duodenum CTIP should be used as test materials in a bioassay using rats, which would measure an increase in pancreatic fluid output, such as that known to be elicited by mammalian secretin and CCK.

Control tissues should consist of regions of gut deficient in active peptides. In the mammalian gut, secretin immunoreactive cells are almost entirely restricted to the duodenal and jejunal mucosa (Larsson et al, 1977), although Chey and Escoffery (1976) have reported them to be also present in the antral mucosa of man, dog and rat. As the antral region of the stomach additionally contains a large quantity of gastrin (Nilsson et al, 1973), which has a CCK-like effect, and would therefore interfere with the rat secretin bioassay, non-antral tissue was chosen as porcine control material. Styela pharynx was also collected and extracted to check that any positive bio-activity was specific to the gut. These control tissues were extracted in the same way as the Styela stomach and porcine duodenum.

MATERIALS AND METHODS

Gut extracts

a) Source of standard: Boots secretin (the generous gift of The Boots Company Ltd) was used as a crude stimulant of pancreatic secretion. This material is extremely impure.

According to data supplied by Boots, each milligram of the batch used (CMG 11789Y) contained approximately 100 ng of secretin, 4 ng of CCK and 180 pg of gastrin. Undoubtedly many other gut hormones were also present. However 'Boots' secretin is a readily available pancreatic secretagogue, separate batches of which do not vary greatly in potency (Stening et al, 1968).

It was felt that it would be a sufficiently accurate standard for the purpose of this pilot study. If the Styela gut extracts were found to have any effect on pancreatic secretion then a request for reliable

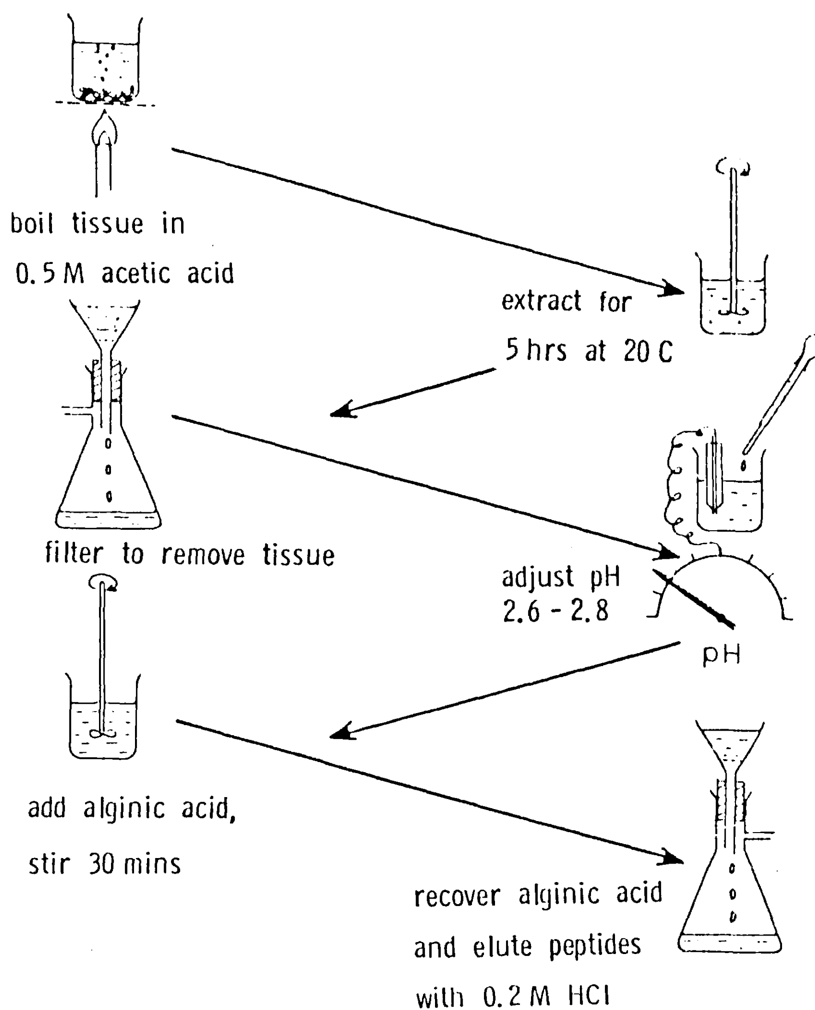


Fig 2:2 The extraction method. Tissues were added to boiling 0.5 M acetic acid, and heated at 80 to 100°C for 10 mins. They were then stirred at 20°C for 5 hrs, after which the extract was cooled, sieved, and finally filtered under reduced pressure. The pH of the filtrate was lowered to between 2.6 and 2.8 by adding 2 M HCl. Carefully washed and fined alginic acid was added to the filtrate and stirred for 30 mins. The alginic acid was recovered and eluted with a small volume of 0.2 M HCl. The pH was adjusted to 4.0 by addition of 1.0 M NaOH. This neutralised solution was then freeze dried.

hormone standards from the Karolinska Institute in Stockholm could be justified.

b) Source of tissues: All porcine tissues used were obtained from the British Beef Abattoir, Watford. Complete gastrointestinal tracts, received within 5 mins of slaughter, were packed in insulated containers with ice and transported to London by van. The first metre of the duodenum and the proximal stomach were removed separately, emptied of their contents, flushed with cold water and minced.

Specimens of Styela clava were collected in the Portsmouth area and transported to London in aerated seawater. The gut and pharynx were excised separately and transferred to dry ice prior to processing.

c) Processing of tissues (Fig 2:2): Extracts were prepared by a method based on the initial stages of the procedures described by Mutt (1959a) and Dockray (1975a). Tissues were added to boiling 0.5 M acetic acid (500 g/l), and heated at 80 to 100°C for 10 mins. Following stirring at room temperature for 5 hrs, the bulk of the solid material was removed by passing the extract through a stainless steel sieve (1 mm mesh). The liquor was cooled and filtered under reduced pressure using Whatman 113 paper. The pH of the filtrate was then lowered to between 2.6 and 2.8 by the addition of 2 M hydrochloric acid.

Alginic acid (Sigma, London) was hydrated in distilled water, fined and then washed with acetic acid (0.5 M), hydrochloric acid (0.2 M), then more acetic acid (0.5 M). This was added to the filtrate (1g/50g tissue) and stirred at room temperature for 30 mins. It was left to allow the bulk of the alginic acid to sediment. The supernatant was decanted and the remaining alginic acid sediment recovered by filtration using Whatman GF/B paper under reduced

pressure.

The alginic acid was eluted with a small volume of hydrochloric acid (0.2 M) and the pH adjusted to 4.00 by the addition of sodium hydroxide (1.0 M). This neutralised solution was then freeze dried using a Birchover Instruments freeze drier.

Operative procedure

a) Rats (Fig 2:3): Animals of either sex, weighing in excess of 250 g, were fasted for 24 hrs prior to use. They were anaesthetised with a single intraperitoneal (IP) injection of a 25% (w/v) solution of urethane (ethyl carbamate) in 0.9% physiological saline (5 ml/kg).

They were then transferred to a darkened cage with adequate bedding for 1 hr. If after this period they showed any signs of 'light' anaesthesia, ie whisker twitch, corneal reflex or an elevated respiratory rate (above 100/min) an ether soaked pad was placed near the nose until a deep anaesthesia was achieved.

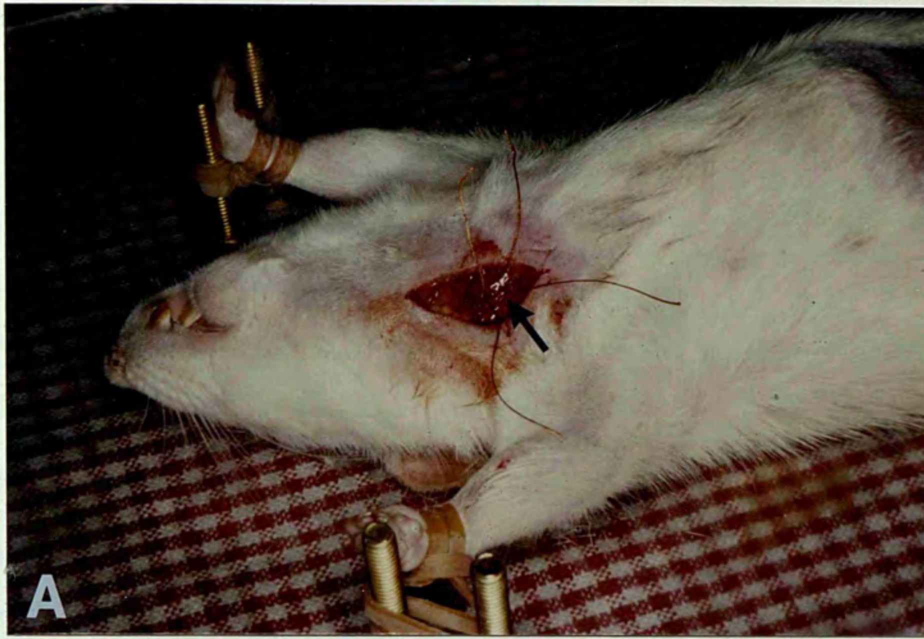
If after the insertion of a venous cannula animals again showed symptoms of becoming 'light' a small amount of the same urethane solution was injected into the cannula.

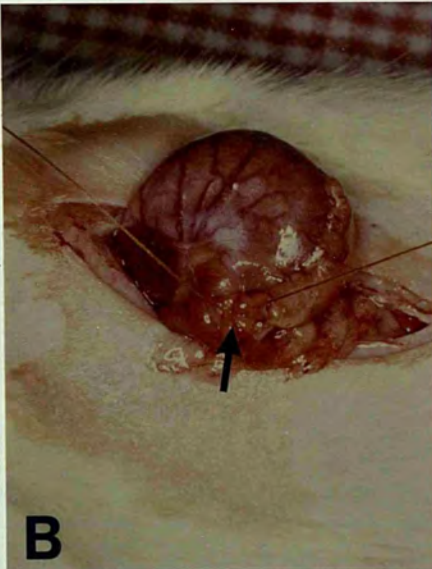
When full anaesthesia had been achieved, the abdomen and neck regions were clipped and the animal was secured to a perspex board using leg ties.

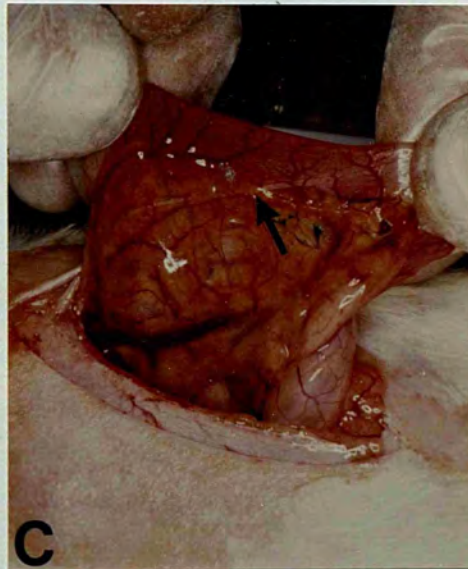
The external jugular vein was exposed and ligated distally (Fig 2:3a). A small incision was made proximal to this ligature and a saline filled polyethylene cannula (Portex pp.00) inserted and tied in place. A saline filled syringe, fitted with a 26-gauge needle was inserted directly into the end of this tube and by alternate injection

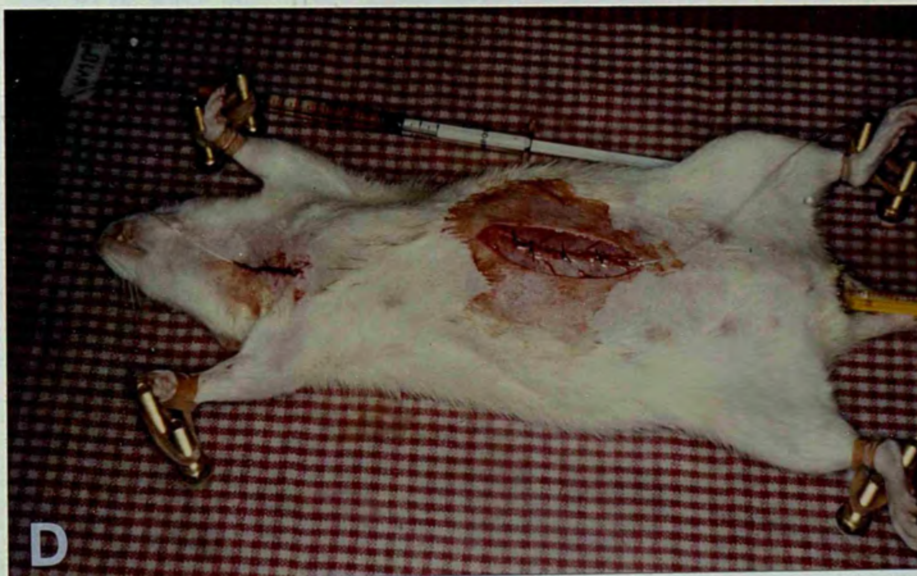
Fig 2:3 The rat bioassay for secretin: a) The anaesthetised rat is shown clipped and secured to a perspex board using leg ties. The external jugular is exposed (arrow), with two ligatures around it. The distal ligature when tightened, prevents the venous return and the proximal one is placed ready to tie in the cannula following insertion. b) The abdominal incision is shown, with the empty stomach withdrawn from the peritoneum. A ligature is shown being drawn tightly around the pyloric sphincter (arrow). c) When the stomach is replaced,the first duodenal loop is withdrawn and the bile duct (arrow) identified. This is ligated distally, and cannulated at its junction with the duodenum. d) When this is complete, the intestine is replaced and the peritoneum is closed,normally with small steel clips, silk sutures are more time consuming to insert. The saline filled syringe can be seen attached to the venous cannula. The pancreatic catheter is lead from the peritoneal incision to a glass measuring capilliary. A small 0-50°C thermometer is seen to be inserted into the rectum.

Fig 2:3









and withdrawal a visual check could be made of the correct insertion of the cannula.

This syringe, refilled with saline as necessary, was left connected, except when injections were made, using a similar syringe and needle. Following the injection of test materials a small volume of saline (200 μl), exceeding the volume of the cannula (0.6 μl/cm), was injected to flush out the material from it.

The abdomen was opened with a midline incision from xiphisternum to umbilicus. The pyloric sphincter was identified and ligated to prevent acid passing into the duodenum and causing endogenous secretin release (Fig 2:3b).

In most mammals the pancreas is drained directly into the duodenum by one or two pancreatic ducts. However in the rat a large number of small ducts drain the pancreatic secretions into the bile duct, which for much of its length is embedded in the pancreatic tissue. The pancreatic juice therefore mixes with the bile before passing into the duodenum. However if the flow of bile is interrupted then pure pancreatic juice may be collected from it.

The bile duct was therefore ligated, distal to the lowest biliary radical but proximal to its entry into the pancreatic tissue. A ligature was passed around the distal end of the duct and with the aid of a low power dissecting microscope, a small incision was made in the duct at its junction with the duodenum. It was found to be important that this incision did not sever any of the superficial capillaries, as the subsequent bleeding could cause blood to be introduced into the duct, which occluded the flow from it. The slightly drawn end of a polyethylene cannula (Portex pp.00) was inserted through the incision, and the ligature drawn tight to secure it (Fig 2:3c).

The cannula was led out of the body cavity through the midline incision, which was then closed with braided silk sutures, or small steel clips (Down Bros and Mayer and Phelps Ltd; Mitcham, Surrey). The body, monitored by means of a rectal themometer, was maintained between 36° and 38°C by positioning lights above the animal (Fig 2:3d).

A length of glass capillary tubing (50 µl Drummond 'Microcap') was attached to the free end of the cannula and this was replaced at 5 min intervals. The volume of secretion was assessed by measuring the length of fluid within the tube (1 mm = 0.5 µl).

b) Turkeys (Fig 2:4): Eight week old birds were obtained from a local turkey breeder and maintained on commercial turkey food in an outbuilding. The experiments described were performed on birds weighing 2.0 to 5.0 kg.

The animals were fasted for the 24 hrs prior to being anaesthetised with 20% urethane in 0.9% physiological saline (7.5 ml/kg IP).

They were transferred to a cage with adequate bedding and examined periodically to determine their level of anaesthesia. If after a period of about 1 hr they showed signs of 'light' anaesthesia, ie corneal reflex, they were given a further injection of urethane (0.1 to 0.75 ml/kg IP).

When completely anaesthetised, a wing vein was exposed and ligated distally (Fig 2:4a). A saline filled polyethylene cannula was then inserted into an incision made proximal to this point. This cannula was then used for subsequent injection of test material.

The feathers in the abdominal region were removed and a midline ventral incision made to expose the duodenal loop and pancreas. The

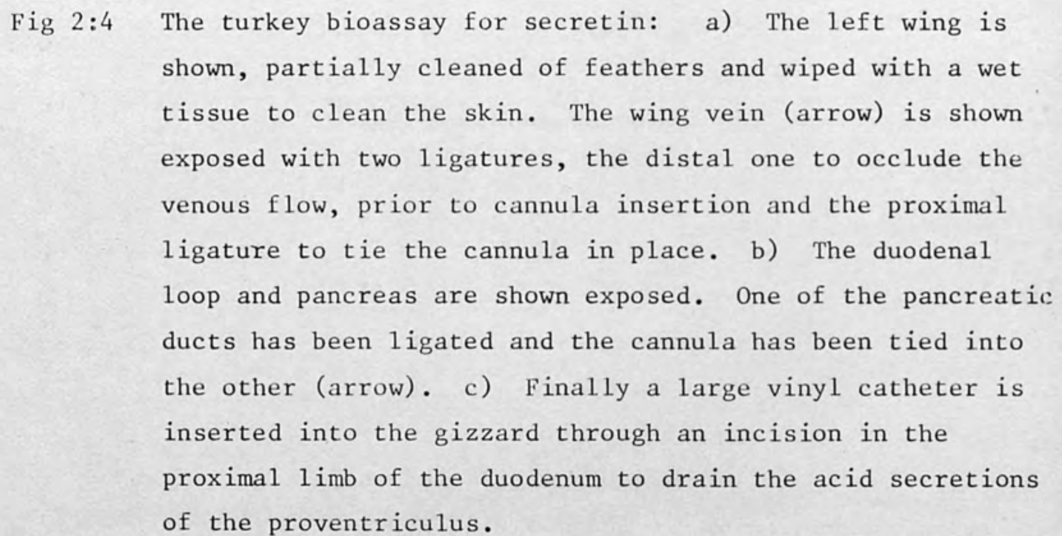
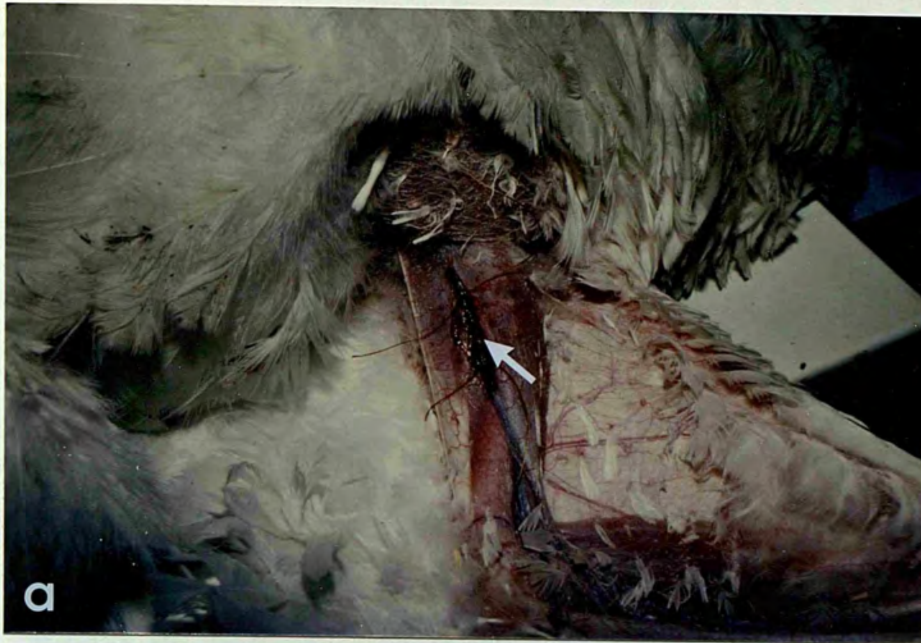
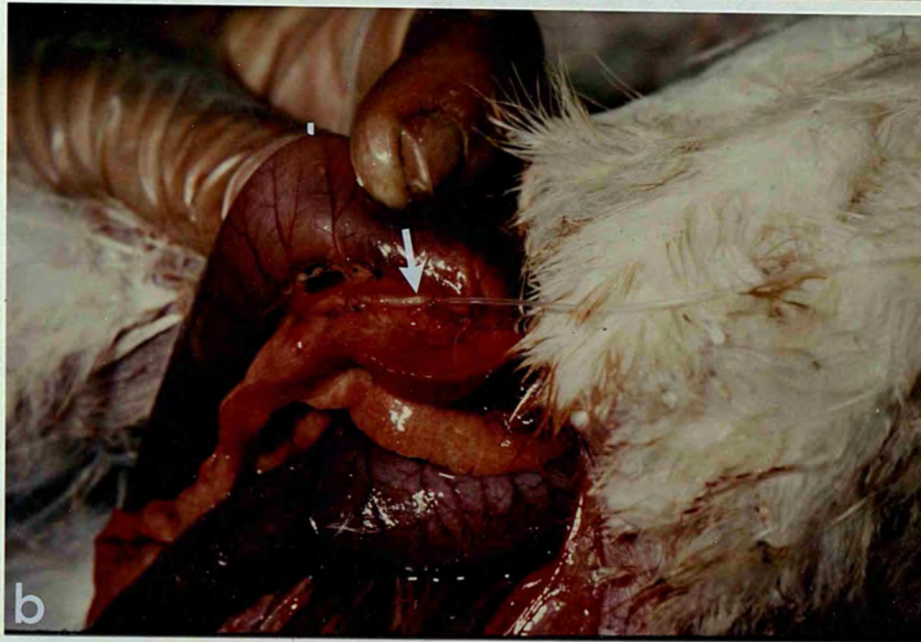
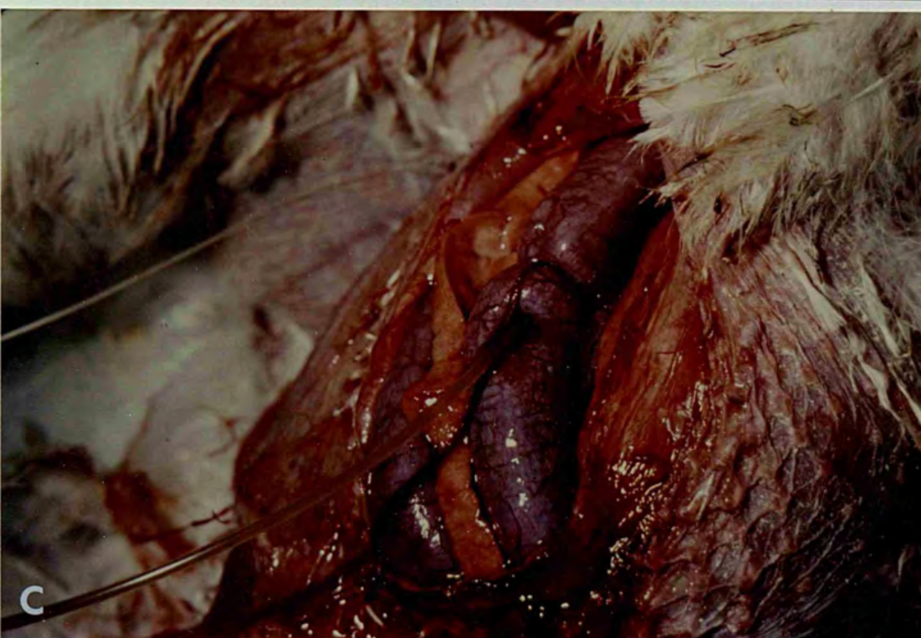


Fig 2:4 The turkey bioassay for secretin: a) The left wing is shown, partially cleaned of feathers and wiped with a wet tissue to clean the skin. The wing vein (arrow) is shown exposed with two ligatures, the distal one to occlude the venous flow, prior to cannula insertion and the proximal ligature to tie the cannula in place. b) The duodenal loop and pancreas are shown exposed. One of the pancreatic ducts has been ligated and the cannula has been tied into the other (arrow). c) Finally a large vinyl catheter is inserted into the gizzard through an incision in the proximal limb of the duodenum to drain the acid secretions of the proventriculus.

Fig 2:4







pancreatic ducts were located. One was ligated at its distal end and a loose ligature was passed around the other at a point immediately before its opening to the duodenum. A small incision was made beyond this point, and a polyethylene cannula (Portex pp.00) was introduced and the ligature tightened to secure it firmly in place (Fig 2:4b). The cannula was led out of the body cavity through a small hole made at one side of the abdomen, situated so as to prevent the cannula from becoming kinked. A vinyl catheter was inserted into the gizzard through an incision in the proximal limb of the duodenum (Fig 2:4c). This drained the acid secretions of the proventriculus, preventing them from entering the duodenum and causing endogenous hormone release.

The abdomen was then closed using a number of steel clips. The body temperature, monitored by means of a rectal thermometer, was maintained between 37°C and 39°C by positioning lights above the bird.

A length of glass capillary tubing (50 µl Drummond Microcap) was attached to the free end of the cannula and this was replaced at 5 min intervals. The volume of secretion was assessed by measuring the length of fluid within the tube (1 mm = 0.5 µl).

<u>Administration of test materials</u>

All hormones and test materials were weighed up on the day of use. They were then dissolved in normal saline containing 0.01 M acetic acid. Injections were made in a random order into the cannulated vein and at least 30 mins was allowed to elapse between injections.

RESULTS

All data obtained from rat and turkey bioassays are presented in tabular form in appendix 2.

The rat

The effect of five doses of 'Boots' secretin on the output from the cannulated rat pancreas is shown in Fig 2:5. Typically, following the injection of a dose of 'Boots' secretin, levels rose from basal (6.05 ± 1.33 µl.5min^{-1}) to a maximum rate of output within 5 to 10 mins. In the case of the highest dose (500 µg/kg), this maximum output was measured as 23.1 µl.5min^{-1}. Larger doses of secretin produced higher maximum outputs, as well as increasing the period over which levels were sustained significantly above basal.

Significant elevations of output were produced by all doses ($p < 0.001$), except for the lowest dose of 1 µg ($p > 0.1$). Peak flow rates occurred 10 to 15 mins after injection.

Within the range of doses studied, the volume of the total response, ie the increase in fluid output caused by the injection, increased with dose (Fig 2:6).

Extracts prepared from porcine duodenum demonstrated an effect on the volume of total response parallel to that of 'Boots' secretin (Fig 2:6). However the extract displayed only about 10% of the activity,ie 500 µg of extract produced a response similar to 50 µg of 'Boots' secretin.

Styela gut extracts produced no significant elevation in the rate of pancreatic secretion when administered as a dose of 0.5 or 1.0 mg ($p > 0.1$). However a dose of 5 mg produced a total reponse which might

TABLE 6

TABLE OF TEST SUBSTANCES AND DOSES GIVEN

RAT BIOASSAY

Material tested	Dose/Weight mg/kg	Number of determinations (n)
'Boots' secretin	0.5	4
	0.05	6
	0.01	3
	0.005	3
	0.001	3
Porcine duodenal extract	1.6	1
	0.8	2
	0.4	2
	0.2	2
	0.1	2
Porcine gastric extract	1.0	4
	2.0	2
Styela gut extract	1.0	4
	2.0	4
	5.0	3
Styela pharynx extract	5.0	4

TABLE 7

TABLE OF TEST SUBSTANCES AND DOSES GIVEN

TURKEY BIOASSAY

Material used	Dose/Weight mg/kg	Number of determinants (n)
'Boots' secretin	0.5	3
	1.0	3
	2.0	3
Porcine duodenal extract	2.0	3
	5.0	3
Porcine gastric extract	5.0	3
Styela gut extract	1.0	3
	2.0	1
Styela pharynx extract	2.0	3

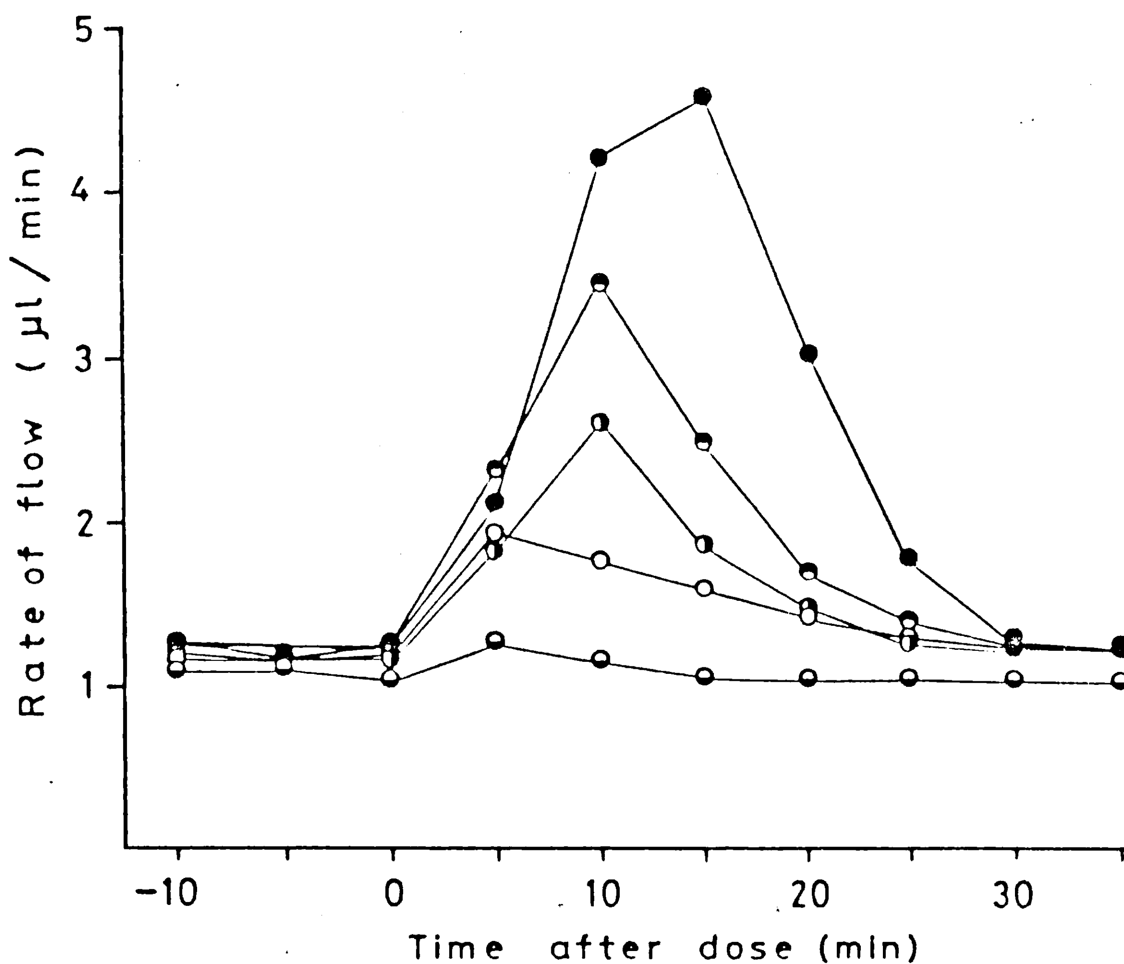


Fig 2:5 The effect of five doses of 'Boots' secretin on the output from the cannulated rat pancreas. Doses were given at time 0 (●, 500 µg/kg; ●, 50 µg/kg; ◑, 10 µg/kg; O, 5 µg/kg; ●, 1 µg/kg).

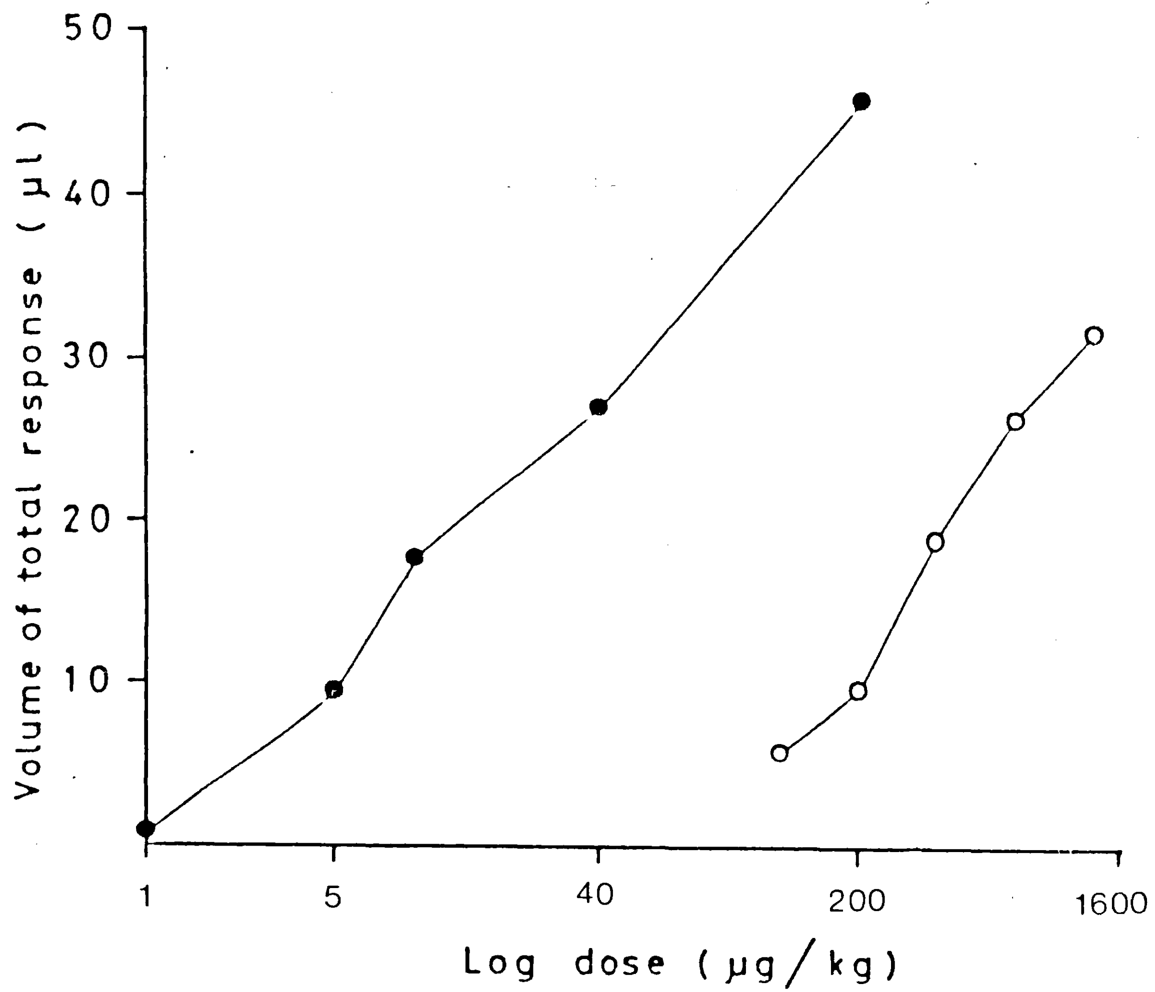


Fig 2:6 The effect of increasing doses of 'Boots' secretin (●), and porcine duodenal CTIP (O) on the volume of the total response in the rat.

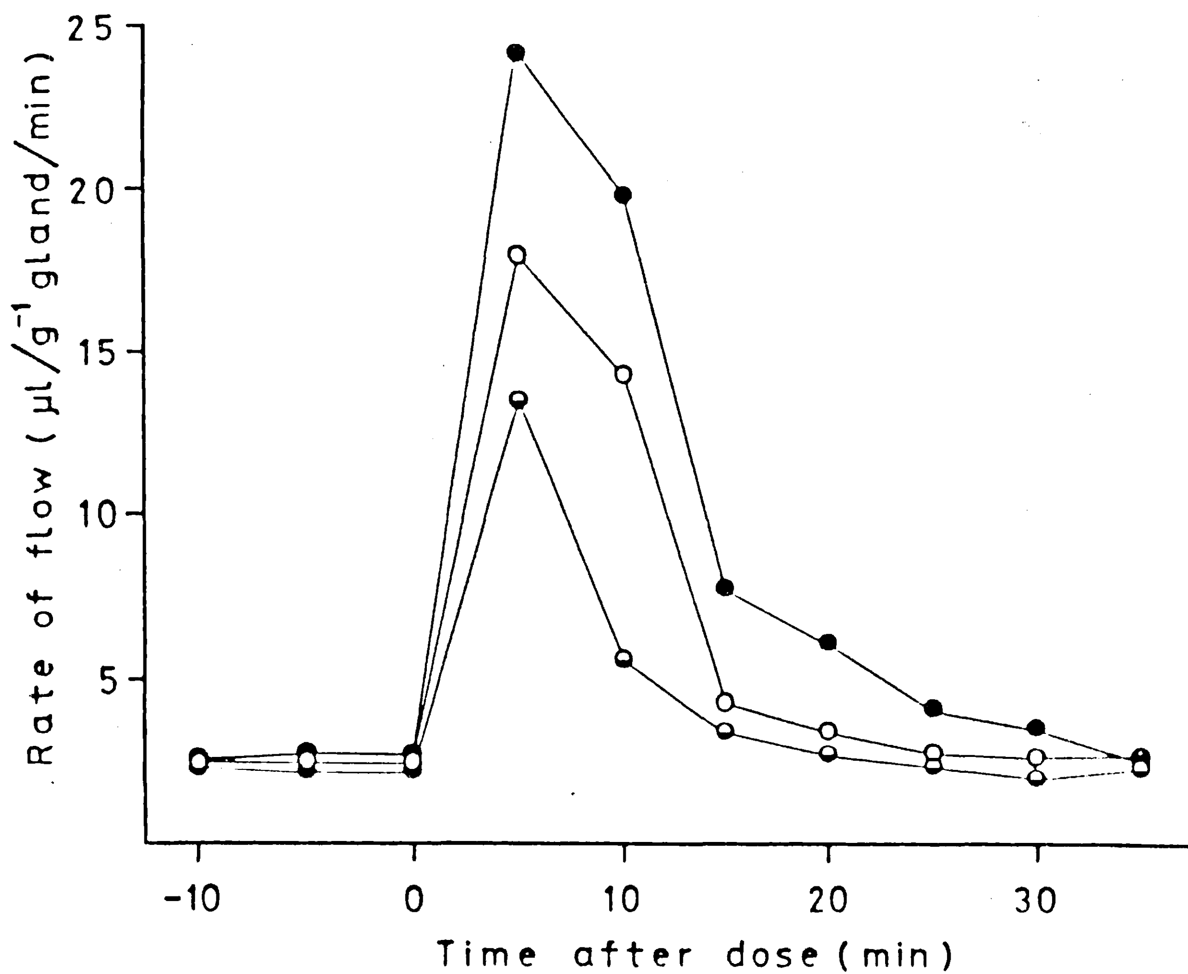


Fig 2:7 The effect of three doses of 'Boots' secretin on the output of the cannulated turkey pancreas. Doses were given at time 0. (●, 2.0 mg/kg: O, 1.0 mg/kg; ⊖, 0.5 mg/kg).

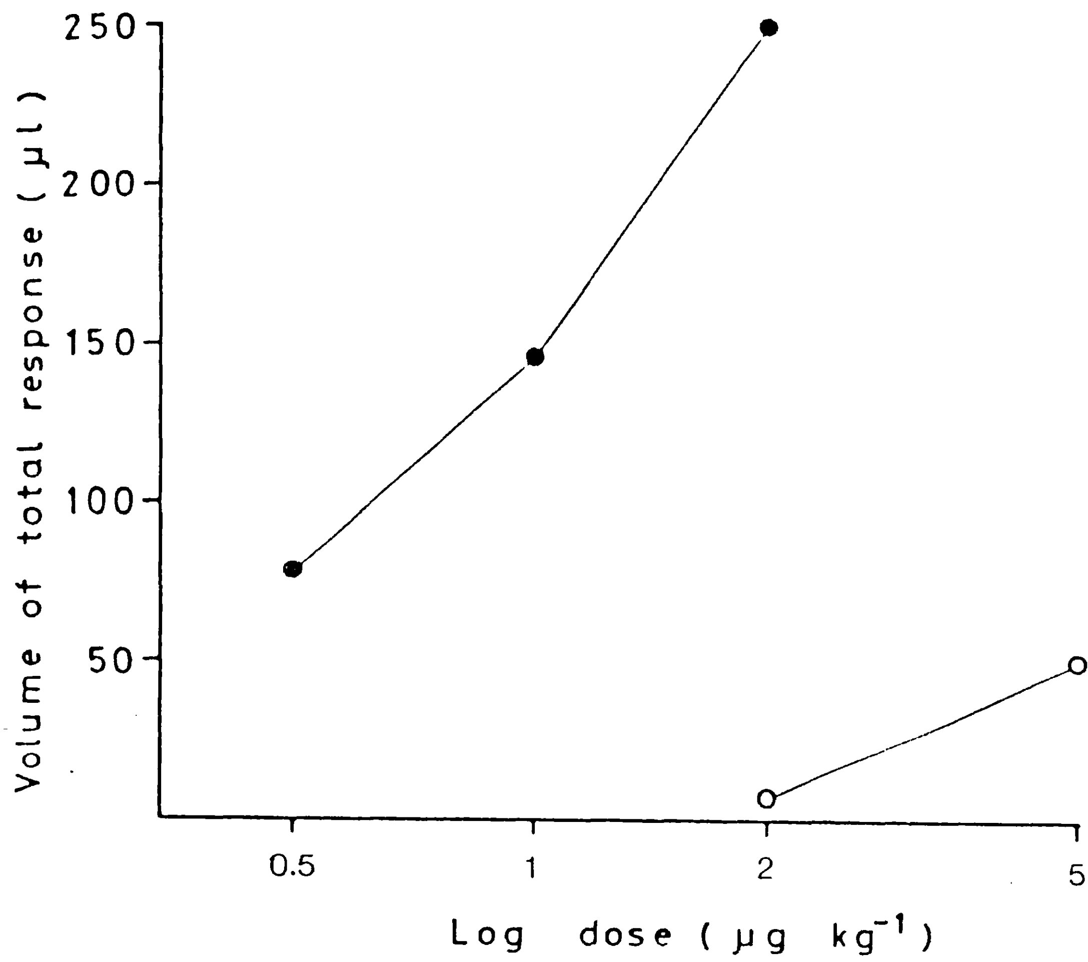


Fig 2:8 The effect of increasing doses of 'Boots' secretin (●) and porcine duodenal CTIP (O) on the volume of the total response in the turkey.

be considered marginally significant ($0.05 < p < 0.1$).

Porcine gastric extracts (1.0 and 2.0 mg) and extracts of Styela pharynx (2.5 mg) produced no significant elevation in pancreatic fluid output ($p > 0.1$).

The turkey

The effect of three dose levels of 'Boots' secretin on the rate of flow from the cannulated turkey pancreas are shown in Fig 2:7. The pattern of activity is the same as that found in the rat (Fig 2:5). Increased doses of secretin caused increases both in maximum output and duration of response. The relationship between dose and the volume of the total response are shown in Fig 2:8.

Porcine duodenal extracts (2.0 and 5.0 mg) both caused significant elevations in secretin ($p < 0.01$) (Fig 2.8). Porcine gastric extract (5.0 mg/kg) produced no response ($p > 0.1$).

Extracts of Styela gut (1.0 and 2.0 mg/kg) and Styela pharynx (2.0 mg/kg) produced no significant elevation in pancreatic output ($p > 0.1$).

DISCUSSION

Both rat and turkey bioassay systems when used to test 'Boots' secretin appear to have operated successfully, as a bioassay, in a dose related fashion. However, insufficient data was collected for any meaningful comparison with the results obtained by other workers who have employed these techniques.

These assays were performed in this instance, not for the accurate determination of specific levels of secretin, but in the hope of

initially establishing whether extracts of Styela gut contained a factor with a secretin-like action on the vertebrate pancreas.

Clearly no such conclusive demonstration has been made. This result however, may not be inconsistent with the immunohistochemical findings reported in chapter 1.

The most likely explanations for this are that either the secretin-like immunoreactive material is not biologically active in the systems used, or that the factor has not been retained in the purification system. Alternatively an inhibitory factor might have been co-extracted, which has completely or partially masked the effect of the secretin.

It is possible that the secretin antisera used in this study were directed against a part of the secretin molecule inessential for bioactivity. This would allow binding to take place with peptides which contained this sequence, but had different amino acids elsewhere in the molecule which reduced or abolished secretin-like bioactivity in the rat and turkey.

The specificity of the antisera used was not established, except that it cross-reacted with porcine secretin, though not with glucagon, VIP or CCK. Antisera have been developed which are highly specific for certain known peptide sequences (Dockray et al, 1981; Rehfeld, 1978), but at the time that this study was made no region specific antisera were available for examining secretin-like peptides.

In the case of some peptide hormones, only a small portion of the molecule may be required for full biological activity. The C-terminal octapeptide of cholecystokinin, CCK-8 (Mutt and Jorpes, 1968a) and the C-terminal tetrapeptide of gastrin, G-4 (Tracy and Gregory, 1964) show

Some members of the secretin/glucagon family of peptides

		1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
Secretin	Por/Bov	his-ser-asp-gly-thr-phe-thr-ser-glu-leu-ser-arg-leu-arg-asp-ser-ala-arg-leu-gln-arg-leu-leu-gln-gly-leu-val-NH_2
Secretin	Chick	his-ser-asp-gly-leu-phe-thr-ser-glu-tyr-ser-lys-met-arg-gly-asn-ala-gln-val-gln-lys-phe-ile-gln-asn-leu-met-NH_2
VIP	Por/Bov	his-ser-asp-ala-val-phe-thr-asp-asn-tyr-thr-arg-leu-arg-lys-gln-met-ala-val-lys-lys-tyr-leu-asn-ser-ile-leu-asn-NH_2
VIP	Chick	his-ser-asp-ala-val-phe-thr-asp-asn-tyr-ser-arg-phe-arg-lys-gln-met-ala-val-lys-lys-tyr-leu-asn-ser-val-leu-thr-NH_2
PHI	Porcine	his-ala-asp-gly-val-phe-thr-ser-asp-phe-ser-arg-leu-leu-gly-gln-leu-ser-ala-lys-lys-tyr-leu-glu-ser-leu-ile-NH_2

Fig 2:9 The amino assay sequence of porcine/bovine secretin, with some other structurally related peptides. Por = porcine; Bov = bovine; Chick = chicken. (After Mutt, 1982a).

the same activities as those demonstrated by their parent molecules.

This however is not the case for secretin (Fig 2:9) where the entire peptide chain appears to be required for any substantial level of pancreatic stimulation. The hexacosapeptide des-histidyl-secretin (secretin 2-27), which lacks only one amino acid residue, has less than 1% of the activity of secretin 1-27, in terms of its ability to stimulate pancreatic secretion in the cat (Mutt and Jorpes, 1967) and dog (Solomon et al, 1977). Another analogue in which the phenylalanine at position 6 is replaced by tyrosine shows only 10% of the activity of secretin (Guiducci, 1974).

The aspartic acid residue at position 3 is also crucial and an analogue in which the configuration of the molecule is affected by the incorporation of this aspartic acid residue in the β-configuration, instead of the α, results in a peptide which shows only 0.6% of the potency of normal synthetic secretin in its stimulation of rate of flow and bicarbonate secretion from the dog pancreas (Ondetti et al, 1970a). Analogues with asparagine (Mutt and Jorpes, 1967), glutamic acid or glutamine (Adler et al, 1980; Wunsch et al, 1977) substituted at position 3 have virtually no secretin-like action. However the very low secretin-like effect on the mammalian pancreas of PHI (Dimaline and Dockray, 1980), in which both His-1 and Asp-3 are conserved demonstrates that residues other than 1 and 3 are also important for activity.

Therefore if Styela 'secretin' were to differ from porcine secretin even by only one amino acid substitution at a critical position, then an extract containing this peptide might show no biological activity.

There is however considerable evidence to indicate that *Styela* secretin-like immunoreactive material does possess biological activity. Extracts with a secretin-like action have been prepared from representatives of many of the vertebrate groups, including the mammals (Jorpes and Mutt, 1970), birds (Dockray, 1975b), reptiles and amphibians (Bayliss and Starling, 1903), fish (Dockray, 1975b; Nilsson, 1970) and cyclostomes (Nilsson, 1973).

All of these are evolutionarily more advanced than *Styela* and therefore no more than hint at the possibility that a recognisable and bioactive secretin-like peptide might also exist in the protochordates. However the presence of a biologically active secretin-like factor in the spiral caecum of *Octopus vulgaris*, originally found by Ledrut and Ungar (1937) and confirmed by Mutt (personal communication) provides evidence that secretin may be phylogenetically older than the protochordates and is therefore circumstantial evidence for its presence in this group.

Recently, and subsequent to the present study, extracts of *Styela* gut have been produced, initially with the assistance of Prof Mutt at the Karolinska Institute and later in London, which are capable of causing the stimulation of water and bicarbonate ion production by the cat pancreas, as well as an increase in fluid output from the rat pancreas (Thorndyke and Bevis, 1983).

Although bovine secretin is structurally identical to the porcine variety (Carlquist et al, 1981) chicken secretin (Nilsson et al, 1980) shows 13 amino acid alterations (Fig 2:9), whilst still demonstrating a secretin-like action in cats and birds (Dimaline and Dockray, 1979).

As the structures of gut hormones have evolved in the same manner as other features which are subject to evolutionary pressures (Van

Noorden and Polak, 1979) it is quite possible that a primitive species such as Styela clava might show as great or greater sequence dissimilarity from porcine than the avian peptide. Chicken secretin could equally well be completely atypical, as the porcine structure appears to be strongly conserved in mammals (Carlquist et al, 1981) and other secretins, including Styela could all be more like the mammalian peptide.

Certain alterations of the molecular structure, particularly those affecting the charge of the molecule, could result in the molecule not being retained in the purification system. A crucial aspect of the purification scheme which was used was the reversible adsorption of the basic peptide onto an insoluble acid, in this case alginic acid, which acts as a cation exchanger. If the Styela secretin were not basic then it might not be retained by the alginic acid at this stage in the purification.

Circumstantial evidence favours the hypothesis that Styela 'secretin' would be retained. Alginic acid has been used to purify a wide range of porcine peptide hormones including secretin (Mutt, 1978), GRP (McDonald et al, 1978), CCK-33 (Jorpes and Mutt, 1959), CCK-39 (Mutt, 1976), CCK-58 (Tatemoto, 1984b), VIP (Said and Mutt, 1972), chymodenin (Adelson et al, 1980), somatostatin-28 (Pradayrol et al, 1978; 1980), PHI (Tatemoto and Mutt, 1981), PYY (Tatemoto, 1982) and galanin (Tatemoto et al, 1983).

In other vertebrate species the alginic acid step has been successful in the purification of human VIP (Carlquist et al, 1982); and PHI (Tatemoto et al, 1984a); bovine secretin (Carlquist et al, 1981) and VIP (Carlquist et al, 1979); and avian VIP, secretin and GRP (Dockray, 1975a; McDonald et al, 1980; Nilsson, 1974; Nilsson et al,

1980). CCK/gastrin-like factors have been extracted from rat, frog, salmon, pike, Chimaera monstrosa, dogfish and hagfish as well as secretin-like factors from pike, Chimaera monstrosa and hagfish (Dockray, 1974; Nilsson, 1970; 1973; Vigna, 1979).

In addition to these vertebrate peptides the secretin-like factor in the spiral caecum of octopus has also been extracted using alginic acid (Mutt, pers com). There was therefore good reason to suppose that Styela 'secretin' would also be adsorbed onto alginic acid and indeed this hypothesis has been confirmed by subsequent successful extractions (Thorndyke and Bevis, 1982).

There is thus strong evidence that the Styela secretin-like immunoreactive material is biologically active and can be retained by the type of alginic acid based purification scheme used in this study. It is suggested that the negative results described in this chapter can be explained only by faults in the execution of the experimental technique, rather than the design of the extraction, which resulted in the retention of insufficient activity for its detection in the bioassays used.

Possible causes of loss of bioactivity

a) Enzyme inactivation: Secretin, like all peptide hormones, is readily denatured by proteolytic enzymes. The problem of extracting thermolabile peptides from enzyme rich tissues such as gut and pancreas are commonly dealt with by extracting at low temperatures to reduce enzyme action. Fortunately secretin, and indeed other gut hormones, are thermostable, and by boiling briefly the enzymes may be inactivated, allowing the peptide to be extracted without losses being sustained due to proteolytic degradation.

It was impossible to predict, except from circumstantial evidence, that Styela 'secretin' would be thermostable, but the subsequent successful extraction by this method indicates that this is indeed the case. Losses may occur, due to proteolysis before the tissue is boiled, or subsequently should the period of boiling be insufficient and proteolytic enzymes therefore remain intact. Losses may also be incurred by prolonged boiling if the thermostability is not absolute.

When preparing Styela extracts, guts were rapidly excised onto blocks of dry ice, which ensured extremely rapid freezing of the tissue. Tissue was accumulated in this manner prior to its transfer to boiling acetic acid. The possibility of proteolytic degradation at this stage seems unlikely.

The porcine tissue was collected by van from an abattoir 90 mins drive from the laboratory. Tissues were received within 5 mins of slaughter. No facilities were available for flushing out the gut or dissecting out the required tissue. The entire gastro-intestinal tract was therefore transported to the laboratory where these procedures took place.

The guts were placed into polythene sacks and packed, with an ice/dry ice mixture, into an insulated container. Although the gut was cool when removed at the laboratory, this cooling may well have been a slow process due to the large bulk of warm tissue and it is possible that losses of secretin, due to proteolysis, took place at this stage.

b) Interface inactivation: The formation of foams and films should be reduced as far as possible as peptides tend to denature at interfaces. Considerable frothing however was allowed to take place during the preparation of these extracts due to their difficult nature. Extraction with boiling acid causes lipids and fats to be dissolved up,

thus making filtration extremely slow. Reduced pressures were therefore used to speed up this procedure.

Under these circumstances foaming occurred and as filtrations could take an hour or more, considerable losses could have occurred. It has subsequently been ascertained that filtration can be speeded up by boiling tissue in water, rather than acid, and then extracting with cold acid. These extracts, when chilled to 0°C and particularly following the addition of 2% Hy-flo, become comparatively easy to filter. The tendency of the filtrate to foam can be reduced considerably by the addition of a few drops of octan-1-ol.

c) **Alginic acid:** This material is produced from a variety of species of marine algae in various countries and by a number of methods. The final product is a linear polymer of high, but variable molecular weight consisting of β-(1-4) linked units of mainly D-mannuronic acid (with some L-guluronic acid), in the pyranose ring form. The ratio of these two acid units is variable and depends upon the source.

Because of the uncertain nature of the product it is possible that the alginic acid used in the present study, obtained through 'Sigma', differs from the material which has been used exclusively by the Karolinska group, which they obtain through Ed Mendell Co, Carmel, NY. If the alginic acid supplied by Sigma has a lower affinity for the secretin than that produced by Ed Mendell Co, this would not necessarily be important, as a larger quantity could be used as adsorbant. However a more important feature of alginic acid used in this way is that a proportion of the peptide is irreversibly bound (Mutt, personal communication). Presumably the guluronic acid units adsorb secretin which is not then eluted with 0.2M HCl. These acid units are then blocked and so if the alginic acid had been recovered

and subsequently reused these losses would have been considerably reduced.

To prevent the possible charge that an apparent Styela secretin was merely residual porcine material being belatedly eluted from pre-used alginic acid, fresh acid was used for each extraction. Losses undoubtedly occurred for this reason and so if the 'Sigma' alginic acid contained a larger percentage of guluronic acid than did that supplied by Ed Mendell Co, then significant losses could have occurred.

To help reduce the losses still further, it is normal practice in Prof Mutt's laboratory to use the minimum amount necessary to adsorb the peptide material. Alginic acid is added stepwise and samples of supernatant are repeatedly tested with an equivalent amount of saturated picric acid. When this addition fails to cause a precipitate then further addition of alginic acid would be to no avail. This useful tip was not known at the time and alginic acid was added in a 'one for the pot' fashion in the mistaken belief that what adsorbs will elute. These factors taken together may have produced considerable losses of material.

CONCLUSIONS

This mainly circumstantial evidence points to the conclusion that the lack of demonstrable secretin-like bioactivity was not due to the absence of a secretin-like factor located within the gut mucosa of Styela. It would appear that the method used was unable to produce a sufficiently active extract to be detected at the doses used.

It is suggested that viewed critically, and with a degree of hindsight, various opportunities existed for losses to have occurred.

It is also possible that the highest dose of Styela gut extract used did have a real effect but lack of material prevented the use of a higher dose which might have confirmed this.

Finally when the experiment was repeated using substantially the same techniques but an improved methodology in a period later than that covered by this thesis, secretin-bioactive extracts were successfully prepared from Styela gut (Thorndyke and Bevis, 1983).

These speculations should not however, overshadow the fact that no evidence has been presented in this chapter which demonstrates that Styela clava contains a secretin-like factor. It was therefore felt that in order to gain such evidence it would be necessary to approach the problem from a direction which would not require the preparation of extracts.

If Styela clava was itself the bioassay then commercially available natural and synthetic mammalian peptides could be tested to determine whether Styela could recognise and respond to them.

CHAPTER 3 - STIMULATION OF GASTRIC ENZYME SECRETION BY REGULATORY PEPTIDES IN *STYELA CLAVA*

INTRODUCTION

- Mechanism of feeding
- The vertebrate pancreatic acinar cell
  - a) c-AMP
  - b) intracellular calcium

MATERIALS AND METHODS

- Animals
- Hormones and reagents
- Perfusion technique
  - a) anatomy
  - b) surgical technique
- Experimental procedure
  - a) chemical assays
  - b) administration of test substances
  - c) statistical analysis

RESULTS

- Effect of gut perfusion
- Effect of infusion of tunicate Ringer
- Effect of CCK and secretin on perfusate protein & acid phosphatase
- Effect of Bt2c-GMP on the response to CCK
- Effect of bombesin
- Effect of caerulein
- Effect of physalaemin
- Effect of glucagon

DISCUSSION

INTRODUCTION

In vertebrates, the hormones which are produced by cells in the gastrointestinal tract are broadly concerned with the regulation of the digestive process (Norris, 1980; Tache, 1984; Thompson and Marx, 1984). For this reason it was supposed that the secretin-like immunoreactive factor in Styela would also, if it acted in an hormonal manner, be involved in some way with the process of digestion. Speculation as to what role it might play requires the consideration of the special feeding habits of ascidians.

Mechanism of feeding (Fig 3.0)

All ascidians are ciliary filter feeders and the mechanism by which they ingest their food has long been a subject of study (Goodbody, 1974; Millar, 1953; Roule, 1884). The particular problems associated with this method of feeding have been fully reviewed (Morton, 1960) and will therefore only briefly be discussed here.

The detritus which is ingested by filter feeders is of a very low nutritive value. This means that large quantitites of this material must be taken into, and moved through, the gut which generally requires that a continuous flow must be maintained. It is also essential that it should have a long transit time through the gut, in order for complete digestion to take place. Movement of fine particles in suspension through the gut by cilia or peristalsis, would be impractical. In order to ingest sufficient food and pass it through the gut, the transit time would be too rapid to allow for digestion and assimilation. The particles are therefore normally removed from the feeding current and compacted into a mucus cord before being passed slowly through the gut.

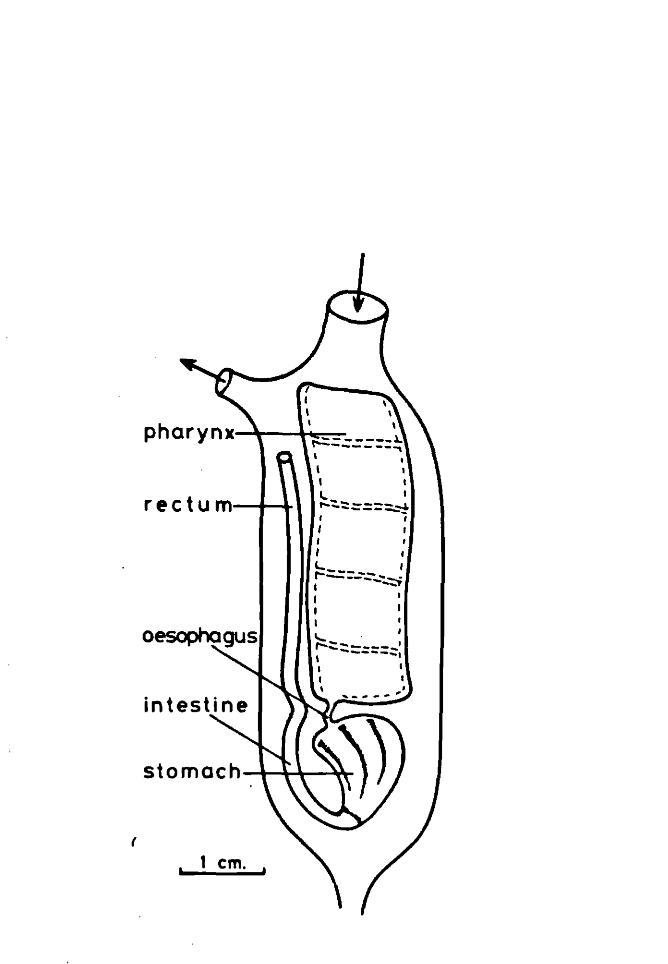


Fig 3:0 The digestive system of the ascidian, Styela clava

In Styela, cilia lining the pharynx draw a current of water through the inhalent (oral) syphon, into the pharynx, and out through a series of slits (stigmata) into the atrium. From there it passes out of the exhalent (atrial) syphon. The inside of the pharynx is covered with a continuous layer of mucus or muco-protein, produced by the endostyle. It is this mucus sheet which traps particles from the water current and in the case of Ciona intestinalis is capable of retaining particles as small as 1.0 μ in diameter (Jorgensen and Goldberg, 1953).

By a combination of ciliary action and waves of contraction of the body wall muscles (Hecht, 1918), the mucus sheet is moved across the branchial wall until it reaches the dorsal lamina. There it rolls into a cord and it is pulled into the oesophagus, by the cilia located at its mouth.

As well as pulling in the mucus food rope from the pharynx, the oesophagus helps to consoldiate it by adding more mucus and giving it a spiral twist. Once in the stomach, the food cord folds upon itself, presumably to increase its transit time. The cord is then drawn into the intestine by the action of a ring of ciliated cells at its stomachic end (Millar, 1953). The food cord is then passed along the intestine by general ciliary action and folded back and forth over the typhlosole, which is an infolding of the intestine that serves to increase its surface area.

Ultimately the faeces are formed by the breaking off of sections of the string, which become coated with mucus in the rectum before being released from the anus into the atrial cavity. They are dispersed through the atrial siphon, by the strong exhalent current.

Knowledge of the digestive processes which occur during the transit of the food string through the gut is extremely rudimentary when compared with the information which is available concerning vertebrate digestion. The rather primitive early studies by Yonge (1925), Berrill (1929) and Van Weel (1940) have been reviewed by Barrington (1962) and more recent studies have been collated by Goodbody (1974).,

Digestive enzymes are secreted into the stomach and digestion appears to be largely extracellular, although there is evidence that some phagocytotic uptake may also occur (Burighel, 1979; Burighel and Milanesi, 1975; Van Weel, 1940; Thorndyke, 1977).

Extracts of gut have been shown to contain enzymes which act as maltase, lactase, amylase, invertase, lipase and protease (Berrill, 1929; Yonge, 1925). However there is no evidence that normal physiological release of these enzymes occurs, although this appears to be a logical inference.

More recent studies have been able to use more sophisticated techniques and therefore produce more meaningful results. Koch and Marsh (1972) have demonstrated that a wide range of polysaccharidases can be extracted from the pyloric gland of *Pyura stolonifera*. These are able to degrade a variety of substrates, including starch, carboxymethyl cellulose and alginates. Elyakova (1972) has confirmed that ascidians, including *Styela clava* can hydrolyse carboxymethyl cellulose and in the case of one species, *Halocynthia aurantium*, a chitinase is also present in pyloric gland extracts.

The pyloric gland was originally thought to have an excretory function (Colton, 1910) but it is perhaps more likely to serve a function analogous to the molluscan hepatopancreas, releasing digestive

enzymes into the gut. The gland consists of a series of tubules terminating in small swellings or ampullae, which ramify over the gut between the internal gut epithelium and the connective tissue sheet which encloses it. The tubules join together and discharge from a single duct which connects to the gut lumen by a small opening at the junction of the stomach and intestine. This pyloric duct is lined with cilia which beat towards the gut so as to discharge some substance into the gut lumen.

Goodbody (1974) draws attention to the fact that early workers (Berrill, 1929; Van Weel, 1940; Yonge, 1925) did not find evidence of digestive enzymes in ascidian intestinal extracts which would have included the pyloric gland. He therefore argues for the pyloric gland having a role in acid secretion, though on rather circumstantial grounds.

In recent years two other workers, Ermak (1975; 1977) and Gaill (1977; 1980) have produced convincing results which indicate an hepatic role rather than a pancreatic role. Ermak (1975) noted that the pyloric tubules occurred directly adjacent to the intestinal absorptive cells and he therefore suggested that this indicated a role in nutrient absorbtion and assimilation. He subsequently (Ermak, 1977) found that the gland cells contained large glycogen deposits, which can be digested with alpha amylase and which are depleted by a period of starvation. He concludes that the ultrastructural, positional and functional characteristics are strikingly similar to those of the vertebrate liver.

The presence of glycogen in the pyloric gland cells of _Dendrodoa grossularia_ has been confirmed by Gaill (1980). He also reports preliminary experiments showing uptake of labelled metabolites,

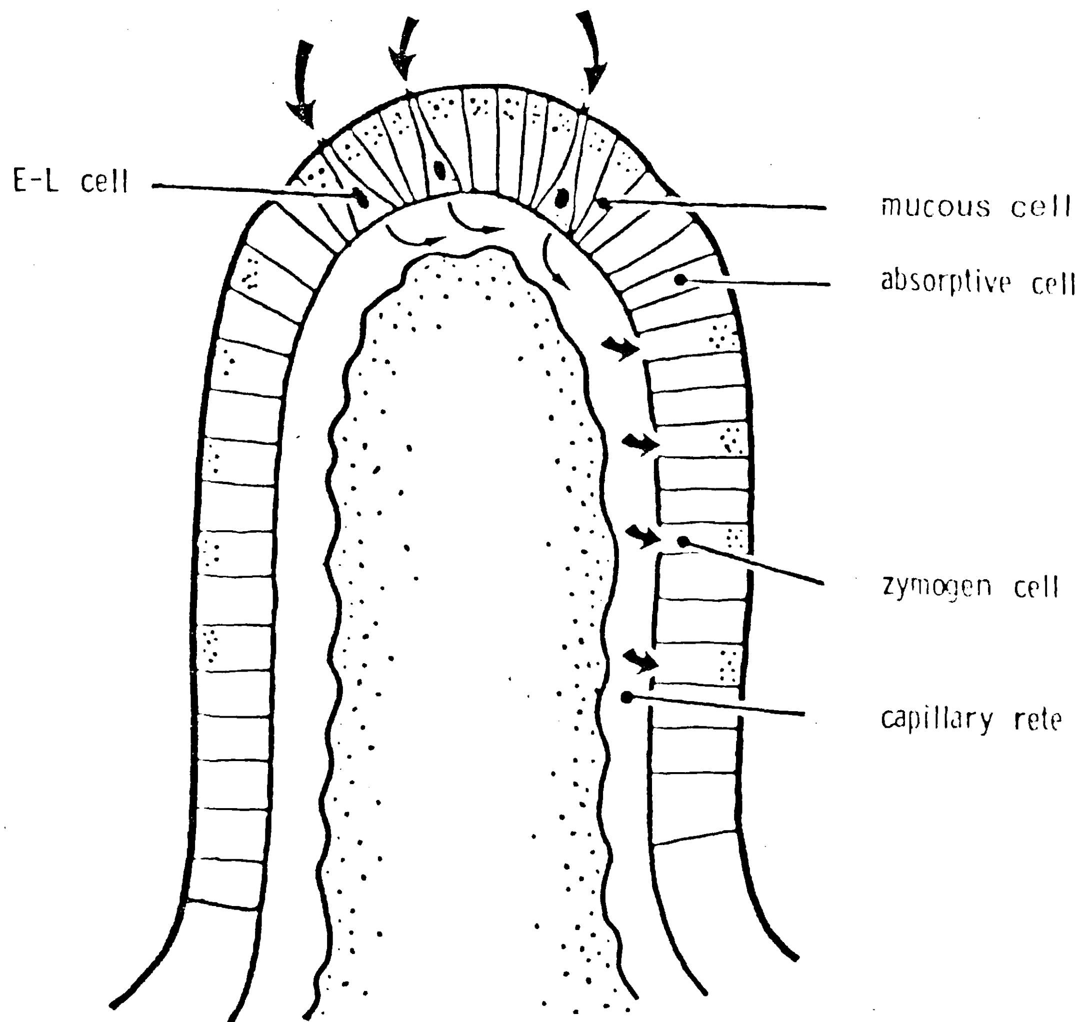


Fig 3:1 Schematic representation of a transverse section of one of the gastric ridges of Styela clava. The cap regions, which consist of mucus cells and endocrine cells, project into the gastric lumen and are therefore well situated to respond to material passing through the stomach. It is suggested that these intraluminal nutrients act as a stimulus, causing the release of an hormonal factor into the underlying capillary rete. This is presumed to stimulate basal receptors on the zymogen cells, situated amongst absorptive cells in the side walls, causing them to release digestive enzymes into the gastric lumen.

particularly glucose from the digestive epithelium, into the pyloric gland.

If the source of digestive enzymes is not the pyloric gland, then the most likely alternate source is the gastric epithelium. The gastric mucosa in Styela is thrown into 20 to 30 longitudinal folds. One of these ridges is a continuation of the ventral oesophageal groove and comprises ciliated mucous cells of the same type as the oesophageal mucous cells.

This raphe expands into a large bulb halfway down the length of the stomach and continues as the typhlosole in the intestine. The remaining ridges (Fig 3:1) have a cap of mucous cells, contiguous with those of the raphe, oesophagus and typhlosole, which contain scattered E-L cells (Bevis and Thorndyke, 1979; Pestarino, 1982).

Between the ridges are pseudostratified undifferentiated cells which are regions of intense cell proliferation (Ermak, 1975; Relini Orsi, 1968; Thorndyke, 1977) giving rise to the cells of the side walls. These side wall cells are of two types. A vacuolated cell, characterised by a large number of apical vacuoles and a protein secreting zymogen cell. The vacuolated cells are however more numerous and undoubtedly have an absorptive function (Berrill, 1929; Burighel and Milanesi, 1973; Morton, 1960; Pestarino and Staffieri, 1980; Relini Orsi, 1968; Thomas, 1970; Thorndyke, 1977; Van Weel, 1940) although Peres (1943) describing possibly corresponding cells in several species of Oikopleura has attributed a secretory function to them.

Experimental evidence of the correctness of the former view has been provided in Styela clava by Thorndyke (1977) and in Botryllus schlosseri by Burighel (1979). They have demonstrated that the absorptive cells of Styela and Botryllus are capable of phagocytosing

ferritin particles and horseradish peroxidase from the gut lumen.

The zymogen cells, with their presumed role of protein secretion are directly pertinent to the present study. Relini Orsi (1968) in her full description of the gut epithelium of Styela plicata, notes the presence of cells secreting protein granules containing tyrosine and tryptophan. She concludes that they probably synthesise digestive enzymes.

Thomas (1970) has described these cells in Ciona as 'gland cells'. From histochemical evidence he concludes that they produce a protein rich seromucous secretion and suggests that they are responsible for the production of the digestive enzymes described by Yonge (1925) and Van Weel (1940). Burighel and Milanesi (1973) have described corresponding cells from the gastric epithelium of Botryllus schlosseri, which they refer to as basophilic or zymogenic cells. They also consider them to play a fundamental role in the production of digestive enzymes.

Corresponding cells in Styela clava, have been described in the side walls of the gastric mucosa (Thorndyke, 1977). They open to both sides of the epithelium with a number of apical microvilli (Fig 3:2a,b). Just below and often closely associated with the apical membrane, are secretory granules (Fig 3:2a). These cells also exhibit prominent rough endoplasmic reticula (RER) and Golgi complexes (Fig 3:2c) which are comparable with the ultrastructural features of protein secreting cells (Barrowman, 1975) and in particular the vertebrate pancreatic acinar cell (Case, 1978; Munger, 1973).

In vertebrates the pancreatic acinar cell produces a minimal output of secretory products under basal conditions but the release of zymogen granule contents is considerably elevated in response to food

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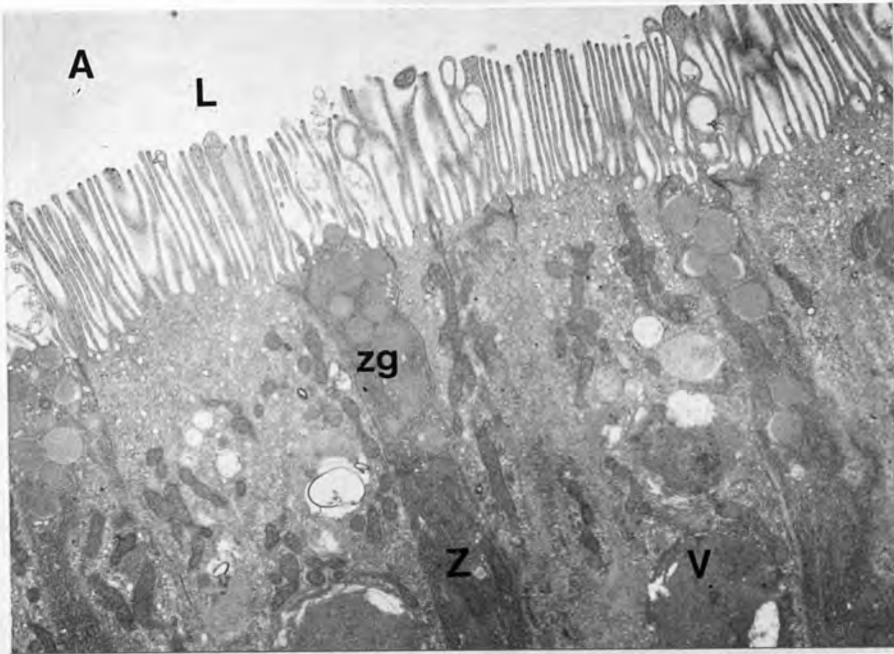
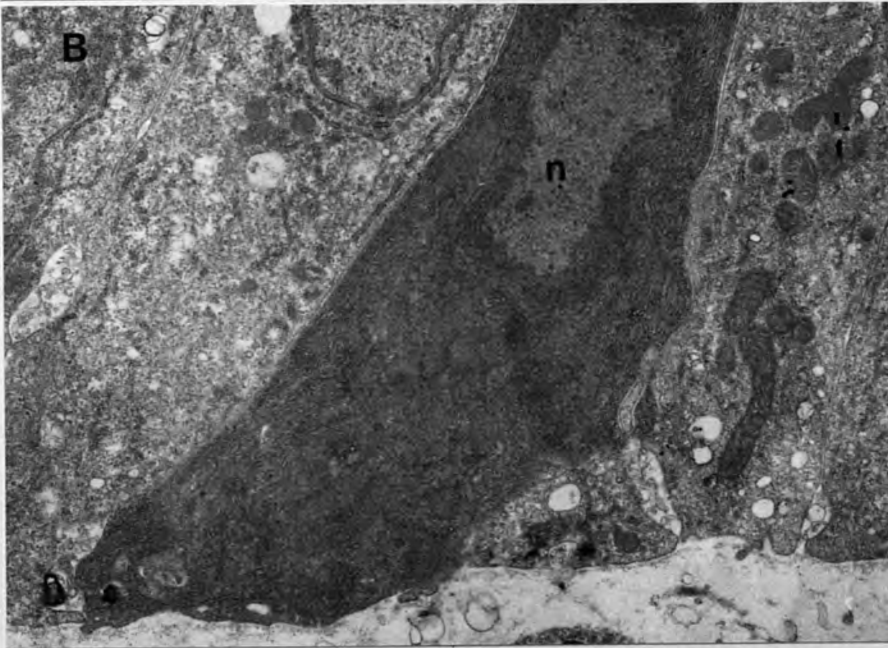
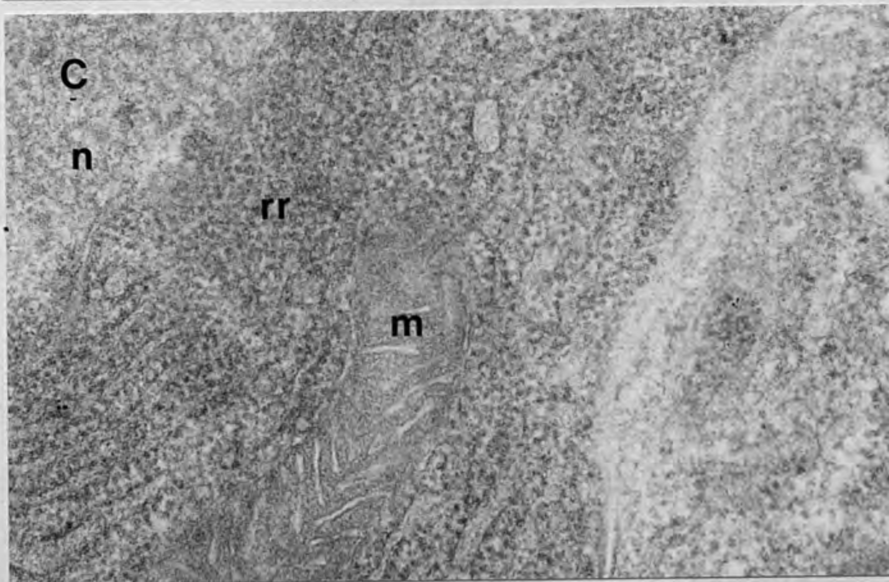
Fig 3:2 Medium and high power electron photomicrographs, showing zymogenic cells between the more numerous vacuolated cells. a) In the apical region the zymogenic cells can be seen to reach the lumen in a constricted region, packed with zymogenic granules. b) In the basal region, large quantities of rough endoplasmic reticulum are evident, in association with mitochondria. These are shown in detail in plate c.
L = lumen; n = nucleus; rr = RER; z = zymogenic cells; zg = zymogenic granules. (a, x 6000; b, x 8750; c, x 50,000)

Fig 3:2







ingestion. This response depends on either acetylcholine (ACh) released at the postganglionic cholinergic nerve endings, or the presence of one or more of a considerable range of peptide hormones.

Since there is no evidence of any gut associated nerves in Styela (Pestarino, personal communication; Thorndyke, personal communication), and considerable evidence indicating the presence of peptide secreting E-L cells, it is possible that the zymogen cells might be controlled in a way which is analogous to the vertebrate system.

The vertebrate pancreatic acinar cell

The function of the pancreatic acinar cell is established as the site of synthesis and release of a wide variety of digestive enzymes including amylase, trypsin, chymotrypsin, lipase and ribonuclease (Desnuelle and Figarella, 1979; Kraehenbuhl et al, 1977). These enzymes are packaged into the zymogen granules and are then secreted by exocytotic release of the granule contents (Case, 1978; Harper and Scratcherd, 1979; Palade, 1975).

Although the synthesis of these enzymes is a relatively continuous process, the secretion occurs episodically as a response to food ingestion. The enzyme secretion is under the control of hormones and neurotransmitters, notably CCK and ACh (Case, 1978; Webster et al, 1977). After stimulation of receptor sites, events are initiated within the cell which result in the controlled release of the zymogen granules from the apical portion of the cell.

Polypeptide hormones and cholinergic agonists, unlike lipid soluble steroid hormones, are unable to enter their target cells due to their size and ionic charge. Instead their effects are mediated through receptor sites located on the external cell membranes. These

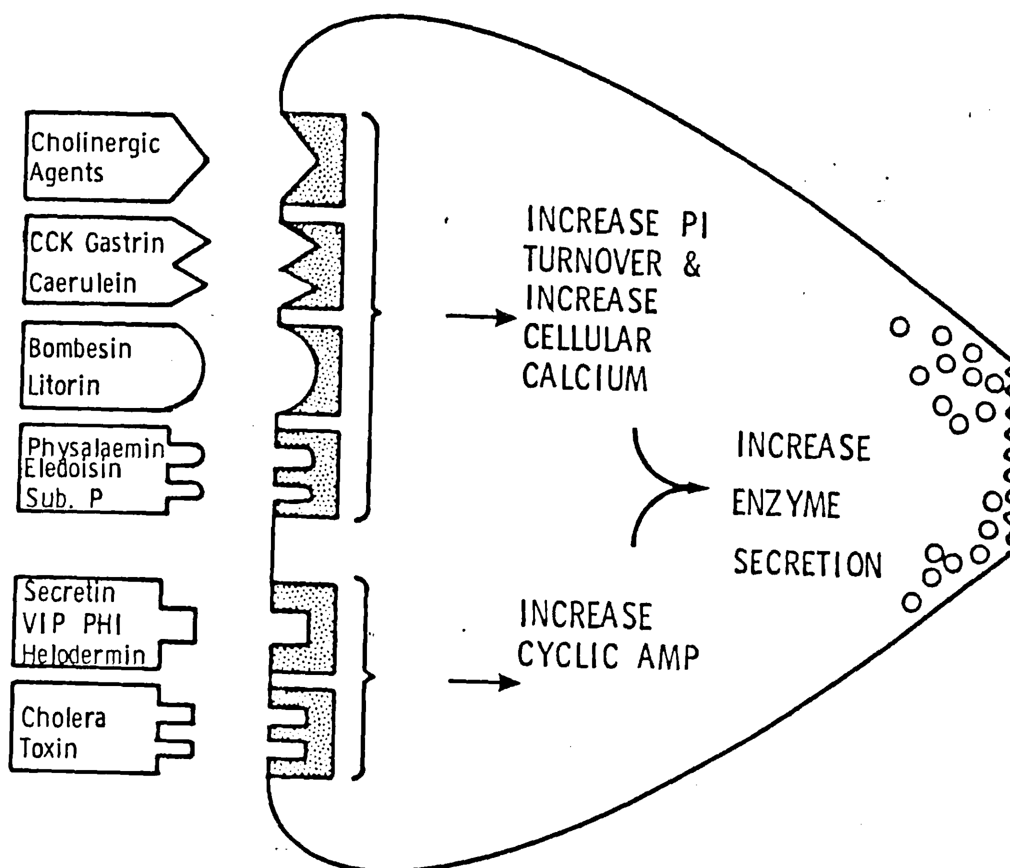


Fig 3:3 Guinea pig pancreatic acinar cells, showing classes of receptors and their mechanism of action. Membrane receptors are activated by various secretagogues to increase enzyme secretion by one of two routes. One route involves an increase in phosphatidylinositol (PI) turnover, followed by an increase in cytosolic ionised calcium: the other involves adenylate cyclase activation, causing an increase in cyclic AMP. Although these two sequences are initially separate, they interact at an unknown point, following the mobilisation of calcium and the generation of cyclic AMP. Potentiation occurs when acinar cells are incubated with a combination of secretagogues which act by different routes. (After Gardner and Jensen, 1983a).

receptors are able reversibly to bind the appropriate ligand, and this initiates the ultimate physiological response by their intermediate effect on some intracellular second messenger system such as 3',5'-cyclic monophosphate (c-AMP).

It has long been known that CCK and ACh operated on acinar cells through different receptors (Harper and MacKay, 1948). However Gardner and his co-workers using radiolabelled secretagogues and dispersed pancreatic acinar cells, have been able to define six functionally distinct classes of receptors which mediate the actions of a considerably larger number of different secretagogues on enzyme secretion (Gardner, 1979a; Gardner and Jensen, 1980; 1983a; 1983b) (Fig 3:3).

These receptors are generally considered to operate via two distinct second messenger systems. One mechanism involves the activation of adenylate cyclase and activation of c-AMP dependent protein kinase, the other mechanism involves the formation of inositol trisphosphate and diacylglycerol from polyphosphoinositide and the subsequent increase of intracellular calcium ions.

a) **c-AMP:** The first group consists of receptors for cholera toxin (Gardner and Rottman, 1979) and for the VIP/secretin/PHI/helodermin group of peptides (Christophe et al, 1976a; 1984; Gardner et al, 1979; Jensen et al, 1981b; 1983; Robberecht et al, 1976; 1984). This second set of receptors may be further subdivided into secretin- and VIP-preferring receptors.

The secretagogue action of Gila monster venom (Raufman et al, 1982) which is produced by the secretin-like peptide helodermin (Robberecht et al, 1984) is mediated through the secretin-preferring receptors. The structurally related peptide PHI, however, induces its

increased enzyme secretion by acting on the VIP-preferring receptors (Jensen et al, 1981a).

Occupation of the VIP-preferring receptors by VIP produces an 8- to 10-fold increase in intracellular c-AMP and a corresponding increase in enzyme secretion. Secretin also increases the c-AMP levels via the VIP-preferring receptors, although its effect is obscured by the larger increase caused by secretin acting on secretin-preferring receptors (Gardner et al, 1979).

Occupation of the secretin-preferring receptors causes an 80- to 100-fold increase in c-AMP, it is not known what cellular function , if any, is effected by this change. Gardner has postulated that there is compartmentalisation of c-AMP and the increase produced by these receptors is not in the appropriate compartment to increase enzyme secretion. Alternatively he has postulated that some acinar cells possess secretin- but not VIP-preferring receptors and that in these cells an increase in c-AMP has no effect on enzyme secretion.

It should also be noted that the increased c-AMP caused by VIP and secretin does not increase enzyme secretion in all species. Robberecht et al (1977) have shown that although VIP and secretin increase c-AMP in fragments of mouse, dog, cat, rat and guinea pig pancreas, enzyme secretion is only potentiated in the guinea pig and rat pancreas.

b) Intracellular calcium: The second group of receptors involve the increase of intracellular calcium ions. Four classes of receptor are known to belong to this group. The cholinergic receptors respond to muscarinic cholinergic agents such as ACh and analogues (carbachol, bethanechol) and are specifically inhibited by atropine (Singh and Webster, 1978).

The CCK receptors are activated by CCK and its various active fragments such as CCK-8, as well as other structurally related peptides such as caerulein and gastrin (Jensen et al, 1980; Sankaran et al, 1982; Schulz and Stolze, 1980). This effect can be specifically inhibited by butyryl derivatives of c-GMP (Peikin et al, 1979) and by proglumide and benzotript (Hahne et al, 1981).

The physalaemin receptors interact with the tachykinins such as physalaemin, kassinin, eledoisin and substance P (Bertaccini, 1980; Jensen and Gardner, 1979).

The final class are bombesin receptors which respond to the structurally related group of bombesin-like peptides, including alyte sin, ranatensin and litorin (Erspamer, 1980; Jensen et al, 1978a; 1978b). These receptors respond in vivo to gastrin releasing peptide (GRP) (McDonald et al, 1979), the mammalian counterpart of these amphibian skin peptides. A substance P analogue, has recently been shown to be a bombesin receptor antagonist (Jensen et al, 1984).

Although these four classes of receptors are clearly very different they are all believed to bring about their actions as secretagogues by the same intracellular mechanisms.

The conformational changes in the receptor, brought about by its activation, cause an increased hydrolysis of certain phospholipids associated with the plasma membrane. The intracellular effects on calcium ion levels and protein kinase C, have been the recent subject of full reviews (Gardner and Jensen, 1983b; Nichizuka et al, 1984) and so will be only briefly described here.

Initially phosphatidylinositol (PI) was identified by Michell (1975) as having an important second messenger role, analogous to

c-AMP, although more recently it has been suggested that polyphosphoinositides which differ from PI by having one (PIP or DPI) or two (PIP2 or TPI) additional phosphate groups on the inositol ring may play a more important role than PI (Marx, 1984).

PI is an acidic phospholipid, normally occurring as a minor membrane component on the inner leaflet of the plasma membrane. When the calcium ion-linked receptors are occupied, PI turnover is initiated by a number of different mechanisms. This turnover is not simply PI hydrolysis but includes increases in synthesis and degradation by a number of mechanisms which have been reviewed by Farese (1983).

This stimulation of PI turnover causes an increase in the ionised calcium concentration of the cytosol compartment of the cell. Evidence that the PI effect precedes the calcium mobilisation is given by the observation that the antibiotic and calcium ionophore A-23187 can mimic the action of the calcium ion-linked secretagogues, without stimulating PI turnover (Calderon et al, 1980).

It has been suggested that the breakdown product, inositol 1,2-cyclic phosphate is directly responsible for this calcium mobilisation (Marx, 1984). The other PI metabolite, 1,2-diacylglycerol, appears to facilitate the activation by ionised calcium of a cyclic nucleotide-independent protein kinase (Kishimoto et al, 1980).

The net calcium ion fluxes are biphasic. The initial requirements are met from calcium ion reserves associated with the plasma membranes and with intracellular organelles. However prolonged stimulation causes a secondary calcium ion influx which is probably due to an increased calcium ion permeability of the membrane and/or an increase in calcium-ion pump activity (Schulz, 1980; Schulz and Stolze, 1980).

The role of c-GMP is unclear, although pancreatic secretagogues which increase cellular calcium also increase c-GMP (Webster et al, 1977; Williams, 1980). The increase in intracellular calcium appears to be directly reponsible for the rise in intracellular c-GMP (Christophe, 1976b), presumably mediated by stimulation of guanylate cyclase production (Schultz et al, 1973). It is possible that this c-GMP causes activation of c-GMP dependent protein kinases (Jensen and Gardner, 1978), and thus has some effect on enzyme secretion. Gardner and Jensen (1983b) do however point out that although CCK increases c-GMP, calcium outflux and enzyme secretion, sodium nitroprusside, whilst causing a 70-fold increase in c-GMP, does not increase calcium outflux or enzyme secretion. It is therefore most unlikely that endogenous c-GMP has a direct effect on enzyme secretion, and most investigators have found that exogenous c-GMP derivatives also do not increase enzyme secretion (Heisler and Grondin, 1975). The fact that some have (Gardner and Jackson, 1977; Haymovits and Scheele, 1976) may be explained by their ability to inhibit cyclic nucleotide phosphodiesterase, with a resultant increase in cellular c-AMP and enzyme secretion (Gardner, 1979a).

The full list of causal relationships between all these observed events and the ultimate zymogen granule release is far from clear. Although release of cellular calcium increases c-GMP, it may be the change in calcium, not the cyclic nucleotide that is ultimately responsible for initiating the increased enzyme secretion.

Although the two receptor-initiated pathways for enzyme secretion stimulation are initially separate there is some convergence, after the stage of cyclic nucleotide production, as potentiation of enzyme secretion can occur between secretagogues which act through the different second messenger systems (Gardner and Jackson, 1977). The

mechanism by which this potentiation occurs however is not clear.

The situation in the postulated acinar cell homologue in Styela, the gastric zymogen cell, may perhaps be broadly similar, from the point of view of receptors, and/or second messenger systems. Quite possibly the best way for testing this hypothesis would be to use a forward, or direct approach, by measuring the binding of radiolabelled hormones to the putative receptor sites on the epithelial cell membrane (Gardner, 1979b). This type of approach however requires the production of radiolabelled hormones with high specific levels of radioactivity, and appropriate detection equipment.

A second problem with this type of approach, particularly so for the inexperienced investigator, is the ease with which false positive results can be obtained. Binding can take place that is not relevant to the cell being studied, or to a portion of the molecule other than the active site, which does not cause receptor activation.

The alternative 'backward' or 'indirect' approach, where the gross biological effect is observed seems to have distinct advantages, from the point of both technical simplicity as well as ease of interpretation. It was therefore decided to develop a gastric perfusion system using Styela in order to test the effect of a range of peptide hormone secretagogues, with particular reference to secretin, in order to examine their effect on gastric enzyme secretion.

MATERIALS AND METHODS

a) Animals: Specimens of Styela clava were collected in the Portsmouth area and transported to London in aerated seawater. The animals were cleaned in running seawater to remove the considerable quantities of detritus and commensals which were attached to the test and transferred to a circulating seawater aquarium maintained at 10°C to 14°C. Most animals adapted well to these conditions and the small percentage of moribund animals were readily identified by their flaccidity and closed siphons. Also dead animals floated to the surface and could be removed. Death normally ensued when the narrow process which connects the body to the holdfast was damaged during collection. Only animals which were healthy, turgid and actively siphoning a week after collection were used in experiments.

b) Hormones and reagents: Synthetic, non-sulphated CCK-8 and secretin were purchased from Peninsula Laboratories Inc, California and technical grade CCK was purchased from Sigma (London). Sincalide (Kinevac, synthetic sulphated CCK-8) was the gift of Squibb Europe Ltd, Middlesex. Synthetic bombesin, caerulein (ceruletide) and physalaemin were the gifts of Farmitalia Carlo Erba, Milan. Pure natural porcine CCK-33 was the generous gift of Prof V Mutt. Dibutyryl-guanosine 3':5'-cyclic monophosphate (Bt2c-GMP), bovine albumin (fraction V) and bovine/porcine glucagon were purchased from Sigma (London).

'Synthetica' artificial seawater was purchased from Waterlife Research Industries Ltd, Longford, Middlesex.

Perfusion technique

a) Anatomy: The result of injections of latex and 'Bateson's[17] Anatomical Corrosion Compound' have confirmed that the circulatory

system of Styela clava in the region of the gut is essentially the same as that found in other ascidians (Alder and Hancock, 1905; Brien, 1948; Herdman, 1904; Millar, 1953).

The blood vessels themselves are simply channels within the connective tissue. Only close to the heart are they lined with endothelial cells where the large vessels which connect with it are lined with a loose cellular reticulum. The smaller lacunae ramify and anastomose, making an extensive capillary rete.

The most noticeable feature of the circulatory system is the heart, a simple 'V' shaped tube lying on the ventral and posterior edge of the stomach, projecting into the pericardium. The wall of the heart is in fact continuous, along one edge, with the pericardium and can be regarded as a tubular invagination of the pericardial wall. The open ends communicate with the large blood sinuses which lead to the branchial sac, viscera, body wall and test.

The cavity of the heart is undivided and has no valves. The wall is formed of a single layer of epithelio-muscular cells with cross-striated fibres which run around the heart. Waves of contraction pass along the heart, propelling the blood in the same direction. These peristaltic contractions are periodically reversed, thereby reversing the blood flow. This interesting phenomenon has recently been reviewed by Goodbody (1974).

The antero-ventral end of the heart (pharyngeal) gives rise to the ventral vessel which accompanies the endostyle and connects with the transverse bars of the pharynx. The other end (visceral) of the heart connects to a vessel which lies on the right wall of the stomach, the cardio-stomachic vessel (CSV). This vessel soon bifurcates to form an anterior and posterior CSV. The posterior branch supplies the network

of vessels which overlie the intestine.

Both vessels divide to form a dense network of small vessels, which ultimately supply an elaborate rete under the gastric ridges (Fig 3:1). Thus on anatomical grounds the large cardio-stomachic vessel would appear to be the best site for the injection of a test substance in order for it rapidly to reach the gastric epithelium.

Unfortunately the wall of this vessel, despite being the strongest in the animal was not sufficiently elastic to seal around the tip of a syringe needle (30 gauge, stainless steel) and consequently considerable loss of blood occurred.

Trials using glass micropipettes drawn on a Narishige microelectrode puller proved that although extremely fine pipettes could be produced, they had insufficient mechanical strength to penetrate the wall of the CSV. When occasional insertion was accomplished (using a Leitz micromanipulator), the tip was normally occluded by cellular debris and injection was not possible.

It was therefore decided that a polyethylene cannula would have to be tied in place with ligatures. This would have the unfortunate effect of stopping the circulation and thus, in order to ensure that material from this injection reached the gastric epithelium, a comparatively large quantity of fluid would have to be given. From trials using Bateson's resin this quantity was determined as 200 μl. The injection also caused displacement of the blood in the stomachic vessels and therefore injections were given in a tunicate Ringer (appendix 1) to minimise the stress which this might induce.

b) Surgical techniques (Fig 3:4): A left ventral incision was made extending the length of the body cavity, passing through all the layers

of vessels which overlie the intestine.

Both vessels divide to form a dense network of small vessels, which ultimately supply an elaborate rete under the gastric ridges (Fig 3:1). Thus on anatomical grounds the large cardio-stomachic vessel would appear to be the best site for the injection of a test substance in order for it rapidly to reach the gastric epithelium.

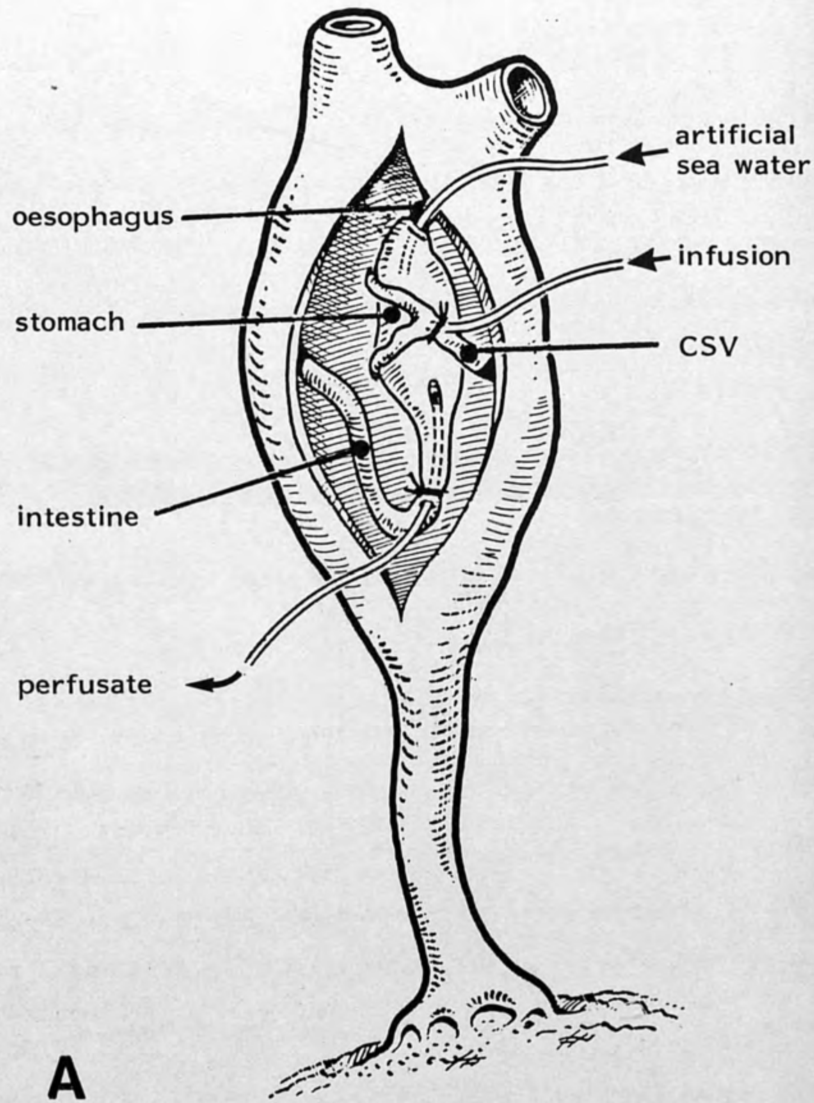
Unfortunately the wall of this vessel, despite being the strongest in the animal was not sufficiently elastic to seal around the tip of a syringe needle (30 gauge, stainless steel) and consequently considerable loss of blood occurred.

Trials using glass micropipettes drawn on a Narishige microelectrode puller proved that although extremely fine pipettes could be produced, they had insufficient mechanical strength to penetrate the wall of the CSV. When occasional insertion was accomplished (using a Leitz micromanipulator), the tip was normally occluded by cellular debris and injection was not possible.

It was therefore decided that a polyethylene cannula would have to be tied in place with ligatures. This would have the unfortunate effect of stopping the circulation and thus, in order to ensure that material from this injection reached the gastric epithelium, a comparatively large quantity of fluid would have to be given. From trials using Bateson's resin this quantity was determined as 200 µl. The injection also caused displacement of the blood in the stomachic vessels and therefore injections were given in a tunicate Ringer (appendix 1) to minimise the stress which this might induce.

b) Surgical techniques (Fig 3:4): A left ventral incision was made extending the length of the body cavity, passing through all the layers

Fig 3:4



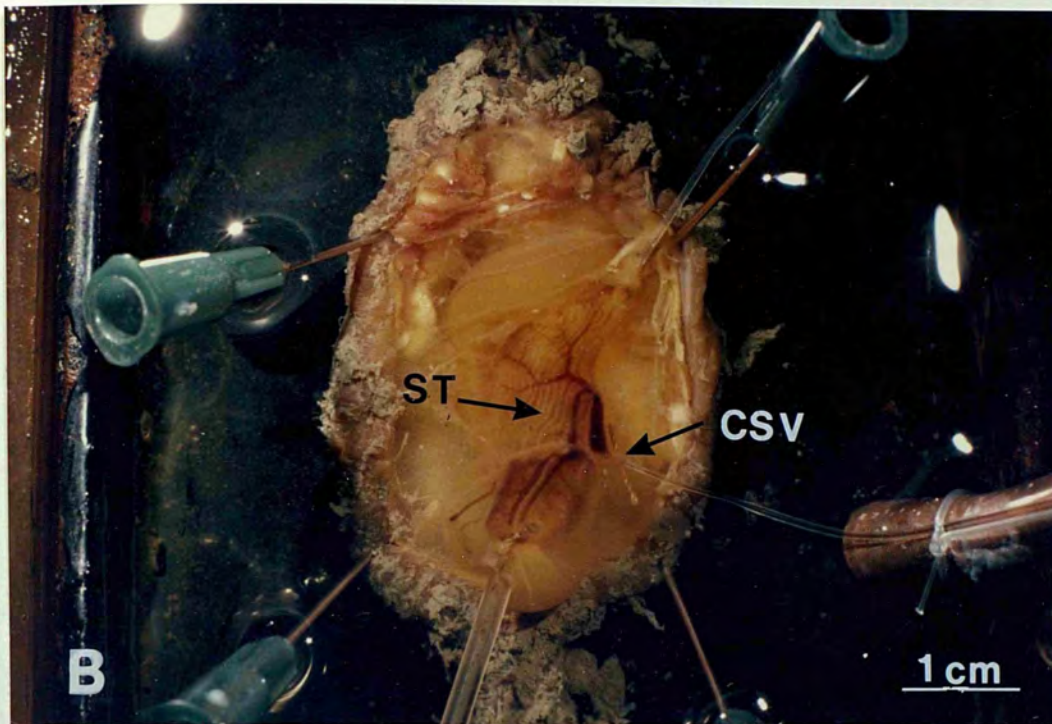


Fig 3:4 The Styela perfusion system. A specimen of Styela clava is shown, opened by a ventral incision, exposing the gut. A small polyethylene tube is shown inserted into the stomach through a small incision in the oesophagus. A larger tube is shown passing into the intestinal end. The stomach is perfused by pumping artificial seawater, at 28 µl/min, into the smaller tube. After passing through the stomach it drains out of the large tube, to be collected prior to analysis. A short polyethylene tube is shown tied into the CSV through which test materials may be infused into the blood vascular system. The system is shown both diagramatically (a) and photographically (b). In (b) a small volume of haemoglobin has been injected into the CSV to make the major gastric vessels stand out clearly. CSV = cardio-stomachic vessel; ST = stomach.

of the test and body wall, so as to expose the gut. A polyethylene tube (Portex 100) was inserted into the proximal end of the stomach through a small incision in the distal end of the oesophagus and secured with a suture. A large tube (Portex 300) was then inserted through a shallow incision on the outer side of the intestinal loop, passing into the distal end of the stomach, and secured by a ligature at the proximal end of the intestine. To prevent the end of this tube from becoming occluded by the gastric epithelium a hole was cut in the side wall of the tube about 5 mm from the proximal end. The animal was then transferred to a small perspex dish through which was passed aerated seawater at 12°C. Using the cannulae, and a 5 ml syringe, the stomach was flushed with 5 ml of filtered seawater to remove any solid gastric contents and then using a binocular microscope the CSV was located and loosely ligated at both ends of the vessel. A small incision was made between the ligatures and a short polyethylene tube (Portex 100) which had been drawn slightly to reduce the outside diameter at one end to 0.2 - 0.3 mm, was filled with tunicate Ringer (internal volume <10 µl) and inserted within the circumference of the stomachic ligature with the aid of a micromanipulator. Both ligatures were then tightened, the stomachic one to tie in the tube and the cardiac one to prevent undue blood loss from the heart.

With practice this procedure took less than 10 min, occasional failures being caused by rupture of the vein during cannula insertion or puncture of the gastric epithelium by the cannula tip.

Experimental procedure

The preparation was maintained in a circulating seawater system at 12°C. Artificial seawater (Synthetica) from a storage tank was passed through a heat exchanger in a tank of coolant maintained at 10°C, into

the dish containing the animal. Surplus water overflowed from this dish, was collected and pumped back to the storage tank. This maintained the water which surrounded the preparation at 12°C (± 1°C).

The stomach was perfused with freshly prepared artificial seawater, prefiltered through Whatman GF/B paper, through the oesophageal cannula, using a Watson-Marlow pump (MHRK-1), at a flow rate of 28 µl/min. The perfusate was collected in the wells of a microtitre plate, the cannula being transferred to a fresh well at 10 min intervals.

After six samples had been collected 200 µl of the tunicate Ringer, containing the test material, was infused through the CSV cannula over a 15 second period.

Samples were taken over a 2 hr period, the infusion being given after 1 hr. At the end of this period, protein and acid phosphatase levels in each sample were determined. At the end of each experiment, 200 µl of filtered, aqueous, methylene blue was injected into the CSV cannula to confirm its accurate placement, and also that no rupture of the delicate vessels had been caused by the original infusion.

a) Chemical assays: Perfusate protein levels were taken as an index of enzyme output and were measured using 200 µl of the seawater perfusate by the method of Lowry et al (1951). This is potentially 10 to 20 times more sensitive than simply measuring the absorbance at 280 nm, more specific and less likely to be influenced by turbidity.

Proteins present in the sample react, after alkaline copper treatment, with Folin phenol reagent, to form a compound which absorbs blue light with a maximum at about 750 nm. This colour may not be strictly proportional to concentration but as values were determined

only within the range of a standard curve, this was not considered significant.

It was found however that the addition of Folin-Ciocalteu reagent (Sigma, London) caused the appearance of a white crystalline precipitate which interfered with the absorbance measurements. Spinning the tubes in a bench centrifuge for the 30 mins development period, however, caused the precipitate to be compacted and a reliable absorbance value of this supernatant could then be determined. This variation of Lowry's (1951) method gave a very reproducible standard curve with values slightly lower than those obtained from protein solutions in distilled water.

Acid phosphatase levels were determined using a colourimetric method with materials supplied by Boehringer Mannheim GmbH, West Germany.

b) Administration of test substances: The weights of individual animals were badly reproducible when repeatedly weighed, as a significant but variable quantity of seawater is retained within the pharynx. A high percentage of the weight of these animals comprised seawater and the keratinous tunic, which was often covered with common fouling organisms. The wet weight of these animals was therefore felt to be an unreliable index for calculating the dose for each animal. Animals were therefore chosen which appeared to be of approximately equal size. Dry weights of most of the animals were determined after use and found to lie within the range 0.51 - 1.22 g.

Test materials were dissolved immediately before use in sterile filtered (millipore GS, 0.22 μ mean pore size) tunicate Ringer (NaCl, 25.9 g/litre; KCl, 0.75 g/litre; $CaCl_2$, 1.05 g/litre; $MgCl_2$, 5.295 g/litre; Na_2SO_4, 1.63 g/litre; $NaHCO_3$, 0.06 g/litre; glucose, 1.0

TABLE 8

TABLE OF TEST SUBSTANCES AND DOSES GIVEN

Test Material	No. of determinations at each dose 0.1 μg	1.0 μg	10 μg	100 μg	200 μg
Bombesin	2	3	4	-	-
Caerulein	-	-	-	3	-
CCK-8 (non-sulphated)	-	-	-	5	-
CCK-8 (Sincalide)	-	-	3	3	8
CCK-8 (Sincalide) + 5 mM Bt_2c-GMP	-	-	-	4	-
CCK-33	-	-	4	5	-
CCK-33 +5 mM Bt_2c-GMP	-	-	3	-	-
Glucagon	-	-	-	4	-
Physalaemin	-	-	-	4	-
Secretin	-	-	-	4	-
Technical grade CCK (Sigma)	0.1mg(4)	0.2mg(5)	0.4mg(4)	1.0mg(5)	

g/litre), prepared from figures produced by Robertson (1954).

c) Statistical analysis: Differences between means were considered to be significant if, using Student's t-test, $p < 0.05$. Values are given as mean ± standard deviation (σ n-1) as determined using a Casio FX-501 P programming calculator.

RESULTS

The levels of protein and acid phosphatase which were determined for each experiment are tabulated in appendix 3.

Effect of gut perfusion

If samples were taken of the fluid normally contained by the gut, it was found to contain high levels of both protein and acid phosphatase. Even after flushing the gut through with seawater, levels were still detectable and experiments were initially performed to determine whether a basal level could be achieved. Fig 3:5 records the effect of perfusing with artificial seawater, a preparation in which the CSV had not been cannulated.

During the first 60 mins, protein levels fell from 10.1 ± 1.5 mg/dl to 2.6 ± 1.5 mg/dl. The levels then remained relatively stable over the subsequent 60 min period.

Acid phosphatase levels showed a similar response. Levels recorded in the perfusate fell from 1.98 ± 0.52 U/l to 0.30 ± 0.12 U/l over the initial 60 min period. The levels did not rise significantly above this value over the subsequent 60 mins.

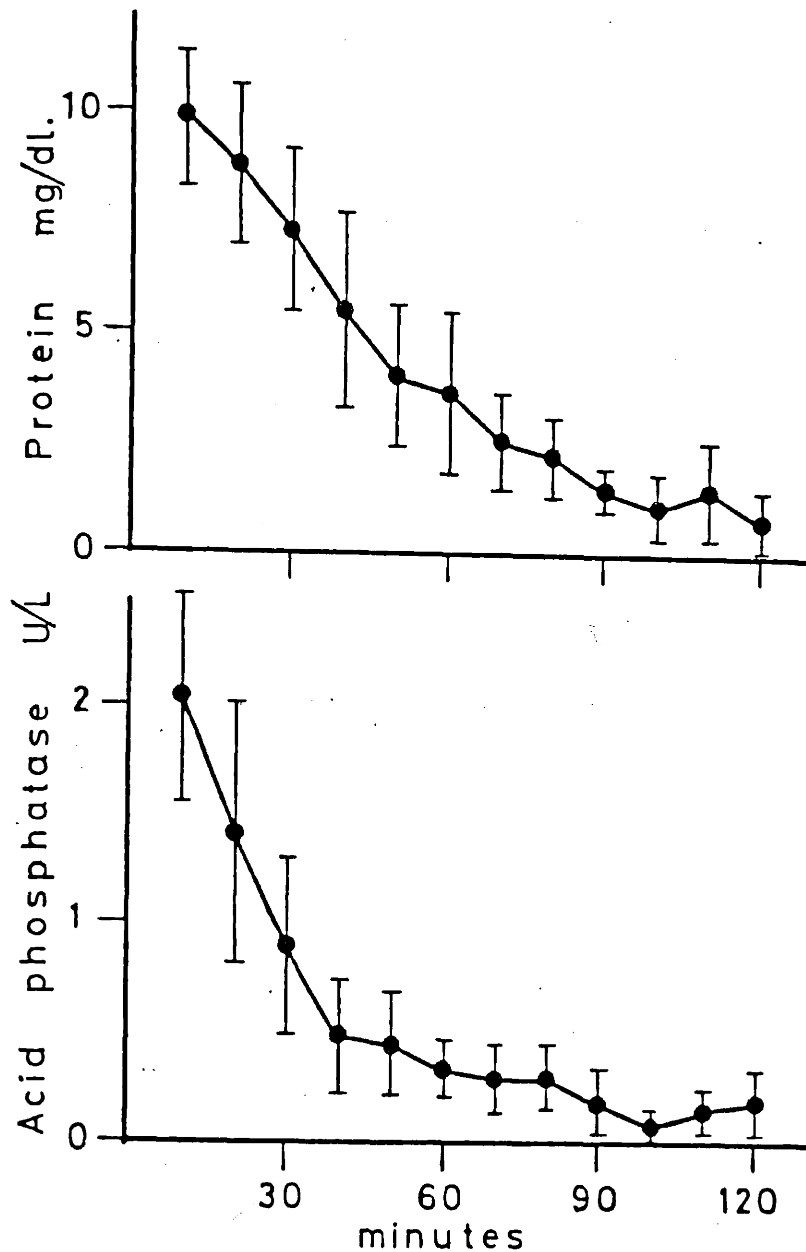


Fig 3:5 Effect of seawater perfusion on protein and acid phosphatase levels in the perfusate over a 120 min period. Mean ± SD, n = 5. Stomachs were preflushed with 5 ml seawater, then perfused at 28 μl/min. During the first 60 mins, levels of protein and acid phosphatase fell sharply, followed by relatively stable levels over the subsequent 60 mins.

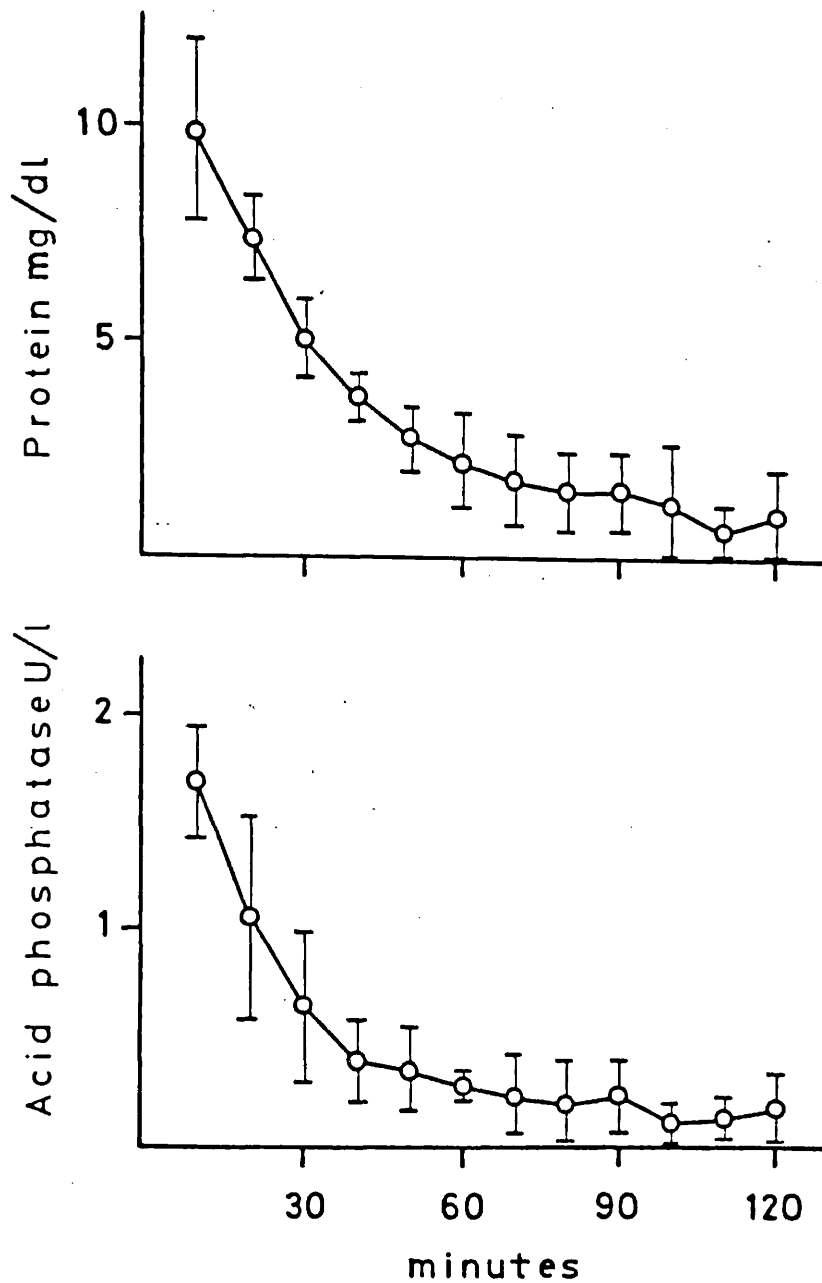


Fig 3:6 Effect of cannulation and subsequent infusion of Ringer on levels of perfusate protein and acid phosphatase. Mean ± SD. The response is not significantly different from that produced by perfusion alone.

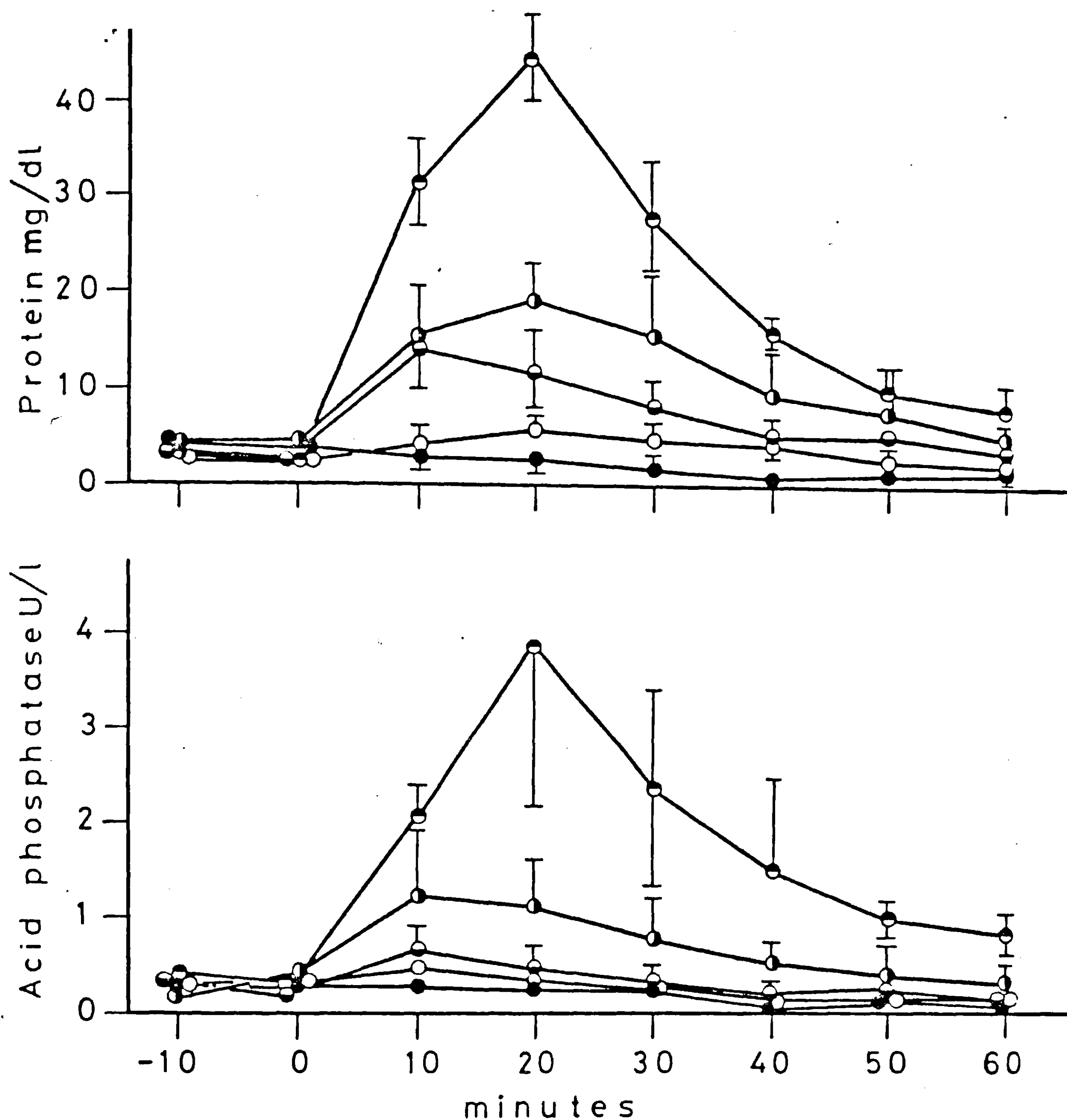


Fig 3:7 Effect of Sigma CCK (infused at 0 min) on protein and acid phosphatase levels in the perfusate over the subsequent 60 min. Mean ± SD. Infusions of 0, 0.1 mg (n = 4), ⊖, 0.2 mg (n = 5), ◑, 0.4 mg (n = 4), and ◐, 1.0 mg (n = 5) CCK all produced levels of protein and phosphatase during the subsequent 60 min which were significantly higher than control infusions (●, n = 6).

Effect of infusion

The effect of cannulation and subsequent infusion of 200 µl of the tunicate Ringer into an animal being perfused as before is shown in Fig 3:6. During the first 60 mins protein levels fell from 9.9 ± 2.1 mg/dl to 2.2 ± 1.1 mg/dl. After the infusion of 200 µl of Ringer, protein levels were not elevated and indeed continued to fall slightly, over the subsequent 60 mins.

Acid phosphatase levels also fell, from 1.68 ± 0.26 U/l to 0.28 ± 0.08 U/l over the first 60 mins. Following the Ringer infusion they showed no elevation and remained fairly constant over the subsequent 60 min period.

The effect of cannulation of the CSV and infusion of 200 µl of vehicle Ringer did not therefore appear to have any effect on the secretion of gastric enzymes.

Effect of CCKs and secretin on perfusate protein and acid phosphatase

Infusion of Sigma CCK at all doses between 100 µg and 1,000 µg produced significant elevations of both protein and acid phosphatase (Fig 3:7). Maximum levels of protein varied from 5.4 ± 1.5 mg/dl, 20 mins after infusing 100 µg, to 42.3 ± 9.6 mg/dl, 20 mins after infusing 1,000 µg.

Maximum acid phosphatase levels ranged from 0.53 ± 0.1 µ/l, 10 mins after infusing 100 ug to 3.85 ± 1.92 µ/l, 20 mins after infusing 1,000 µg.

Recovery to normal basal levels occurred within 60 mins of infusing 100, 200 and 400 µg doses, but 1,000 µg of Sigma CCK produced significantly elevated levels of both protein and acid phosphatase

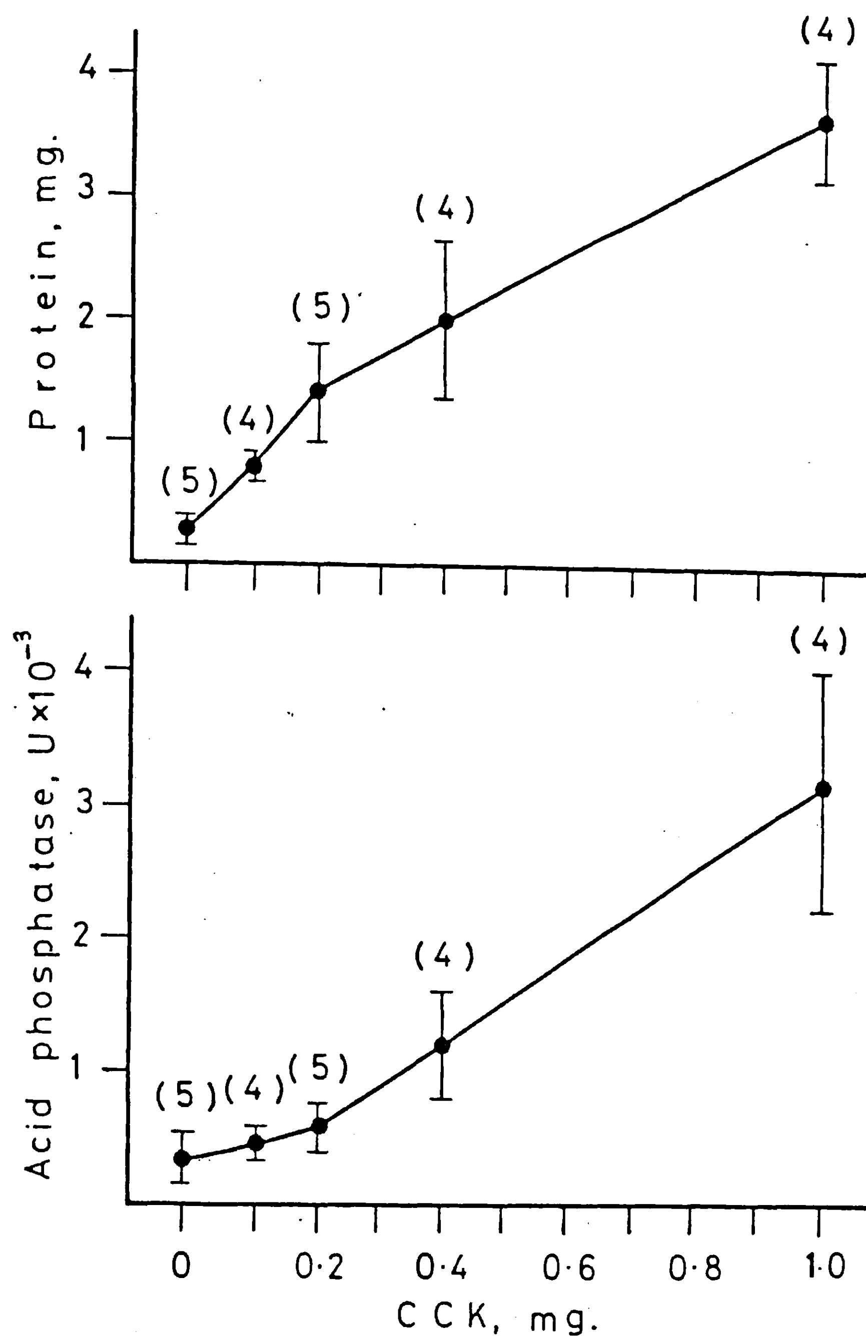
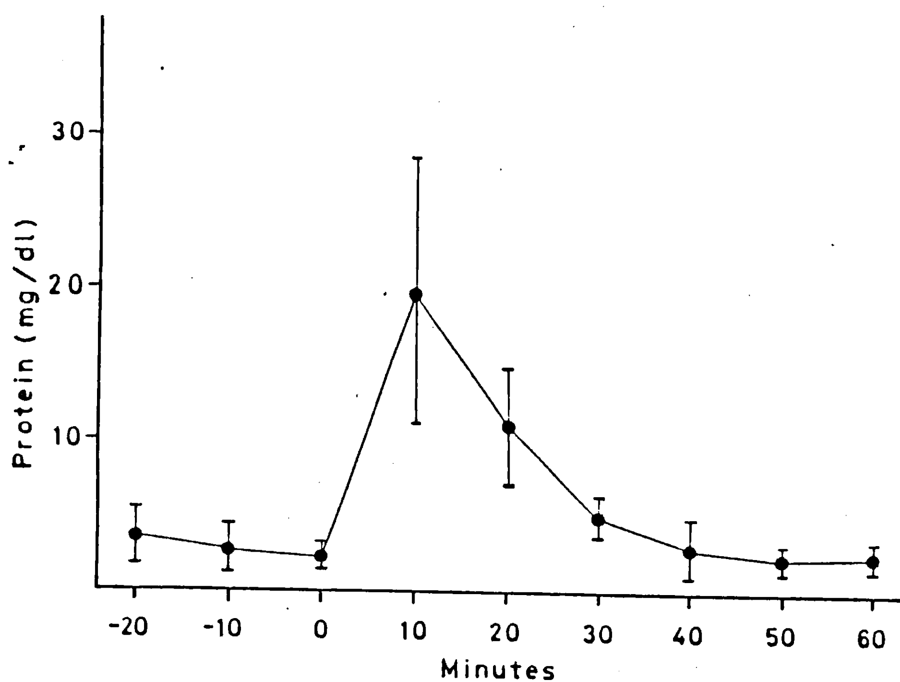


Fig 3:8 Effect on gastric production of protein and acid phosphatase of infusion of Sigma CCK (0.1 - 1.0 mg). Response to each dose is estimated by calculating the total amount of protein and acid phosphatase produced during the 60 min post-infusion period. Mean ± SD. (No. of animals in parentheses). In both instances an approximately linear relationship between log dose and response exists.



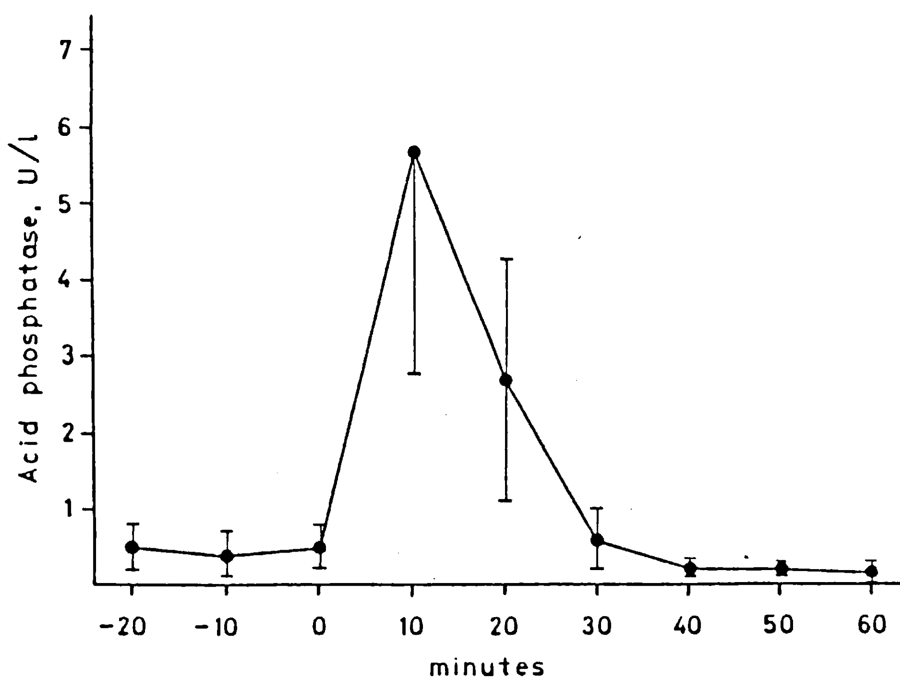


Fig 3:9 Effect of a 100 μg dose of non-sulphated CCK-8 on perfusate protein and acid phosphatase levels. Mean ± SD, n = 5.

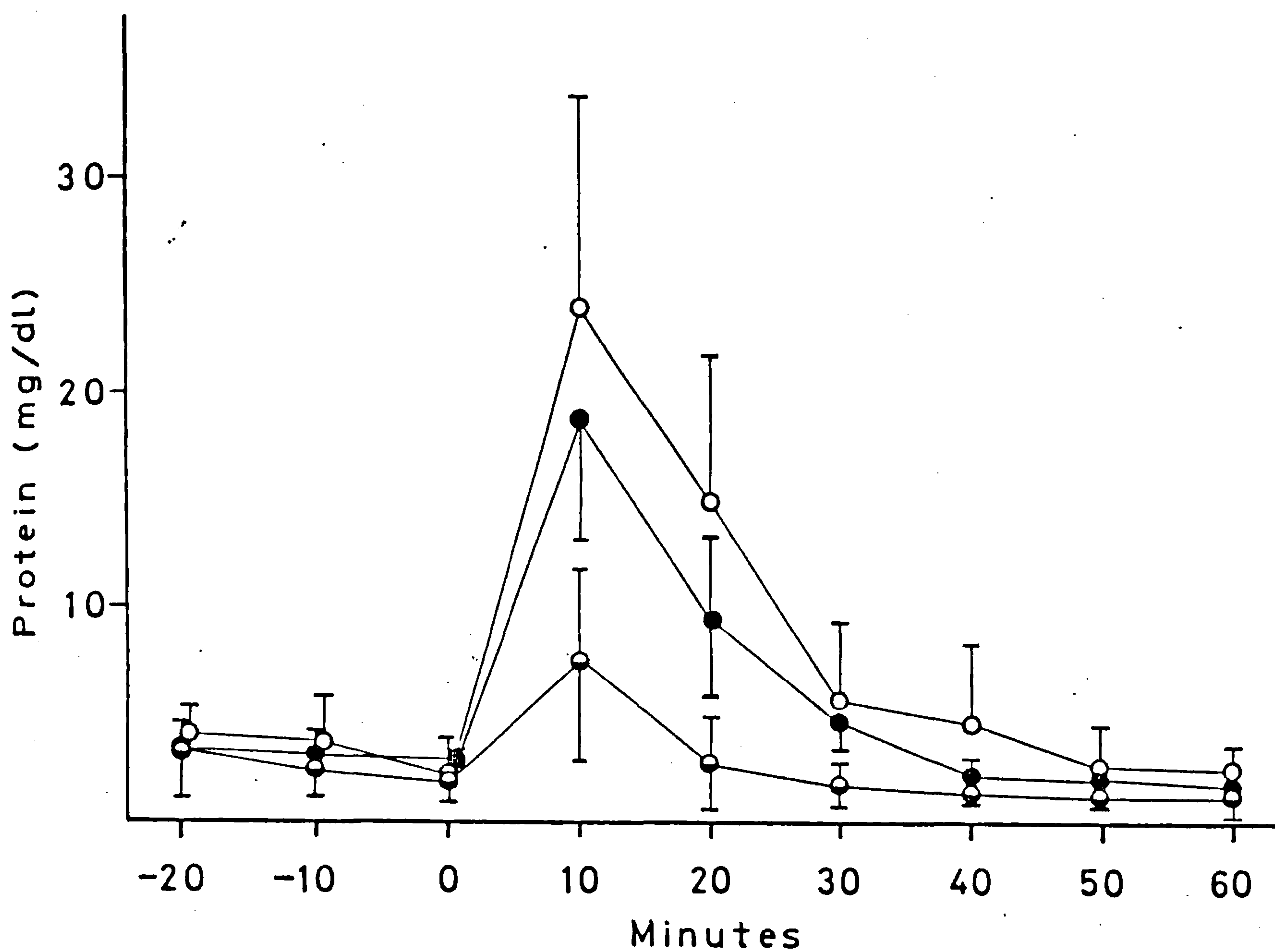


Fig 3:10 Effect of three doses of sulphated CCK-8 on perfusate protein levels. Infusions of 10 μg (θ, n = 3), 100 μg (●, n = 3) and 200 μg (O, n = 8) CCK-8 all produced significant elevations in perfusate protein levels. Mean ± SD.

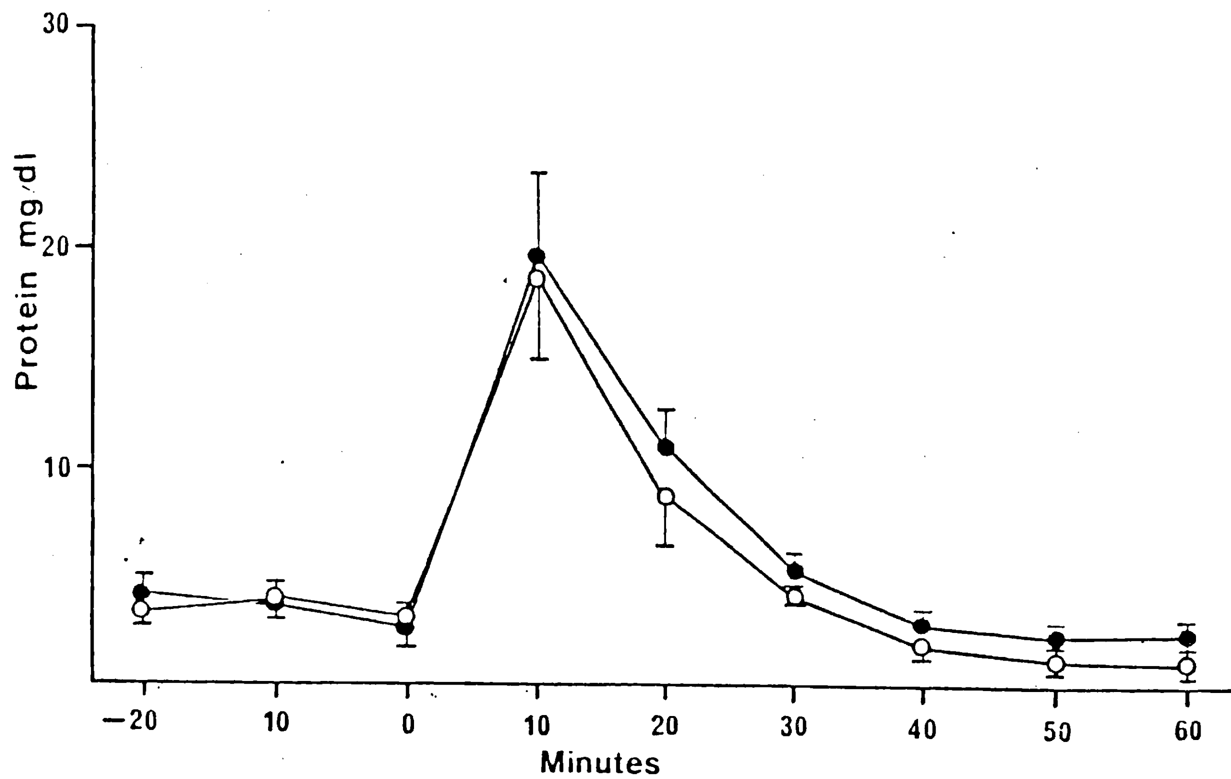


Fig 3:11 Comparative effects of 100 μg of sulphated (●, n = 3) and non-sulphated (O, n = 3) CCK-8. No significant difference in response was observed. Mean ± SD.

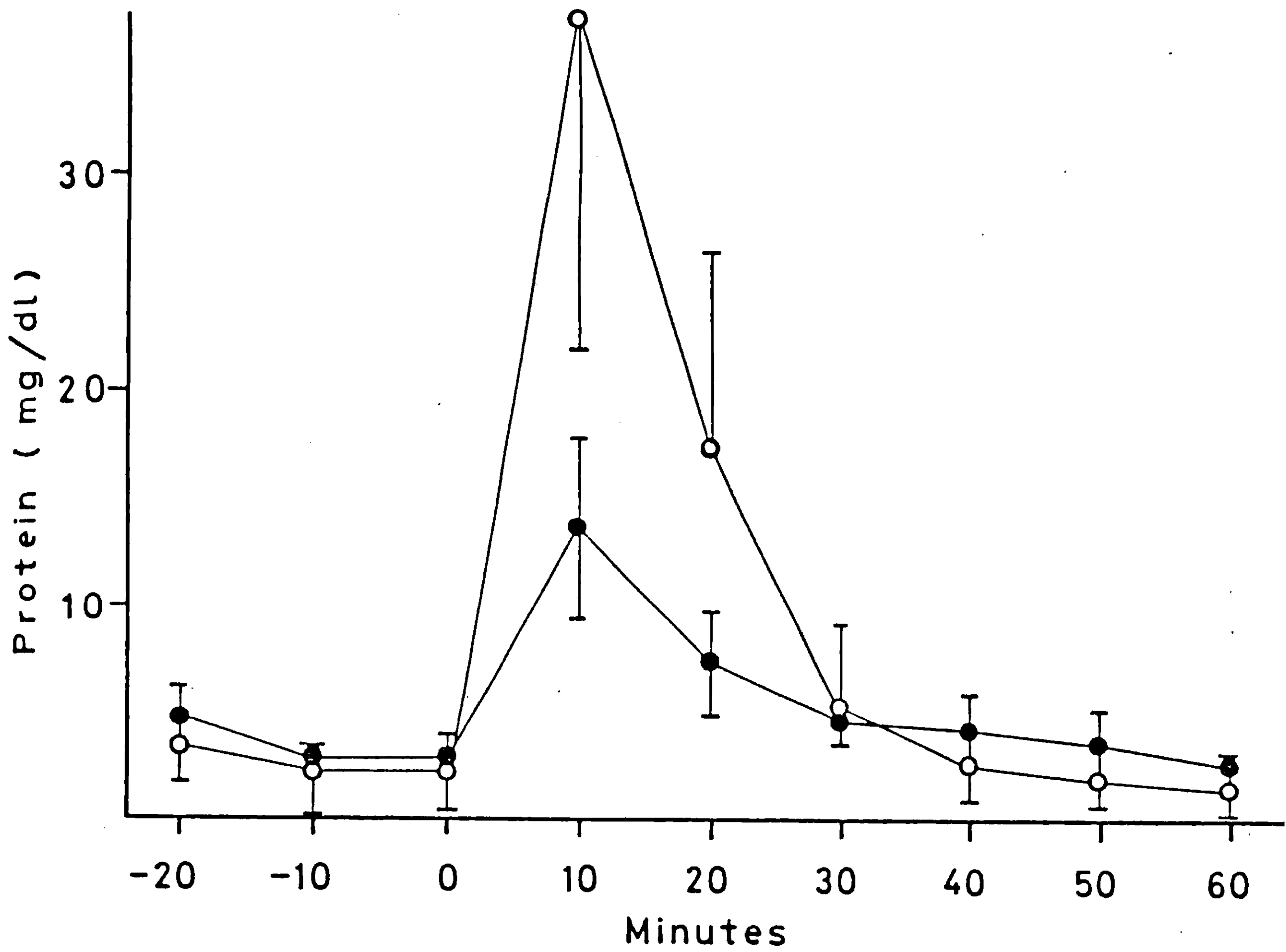


Fig 3:12 Effect of CCK-33 on perfusate protein levels. Infusions of 100 μg (O, n = 5) and 10 μg (●, n = 4) both produced significant elevations in perfusate protein levels. Mean ± SD.

throughout the entire 60 min post-infusion sampling period.

An approximately linear relationship (over the range studied) was observed between the dose of Sigma CCK given and the resultant total output of both protein and acid phosphatase (Fig 3:8). This total output was estimated by calulating the amount of protein or acid phosphatase present in all the samples collected during the 60 min post-infusion period.

Non-sulphated CCK-8 also caused an elevation of protein and acid phosphatase levels (Fig 3:9). A 100 µg (9.41×10^{-8} moles) dose produced a maximum level of protein (19.8 ± 8.7 mg/dl) and acid phosphatase (4.9 ± 2.4 U/1) within 10 mins of its infusion.

Following infusion with sulphated CCK-8, a considerably larger response was observed after a 200 µg (1.75×10^{-7} moles) infusion, than a 100 µg(8.76×10^{-8} moles) or 10 µg (8.76×10^{-9} moles) infusion (Fig 3:10). Whereas a 10 µg (8.76×10^{-9} moles) infusion produced a maximum response of 7.4 ± 4.7 mg/dl after 10 mins, a 200 µg (1.75×10^{-7} moles) infusion produced a maximum protein output of 23.9 ± 10.4 mg/dl after 10 mins.

Similar 100 µg ($8.76-9.41 \times 10^{-8}$ moles) infusions of the sulphated and non-sulphated CCK-8s evoked indistinguishable responses (Fig 3:11). Whereas infusing 100 µg (9.41×10^{-8} moles) of the non-sulphated form was shown to cause a maximal level of 19.8 ± 8.7 mg/dl after 10 mins, falling to 3.0 ± 1.9 mg/dl 30 mins later, an infusion of the same amount of sulphated CCK-8 caused a maximal level of 18.9 ± 6.3 mg/dl after 10 mins, falling to 2.4 ± 0.6 mg/dl after 30 mins.

The larger molecular form, CCK-33, proved to be considerably more potent, particularly when compared on a molar basis (Fig 3:12). A 100 µg (2.55×10^{-8} moles) dose of CCK-33, which in molar terms is only about a

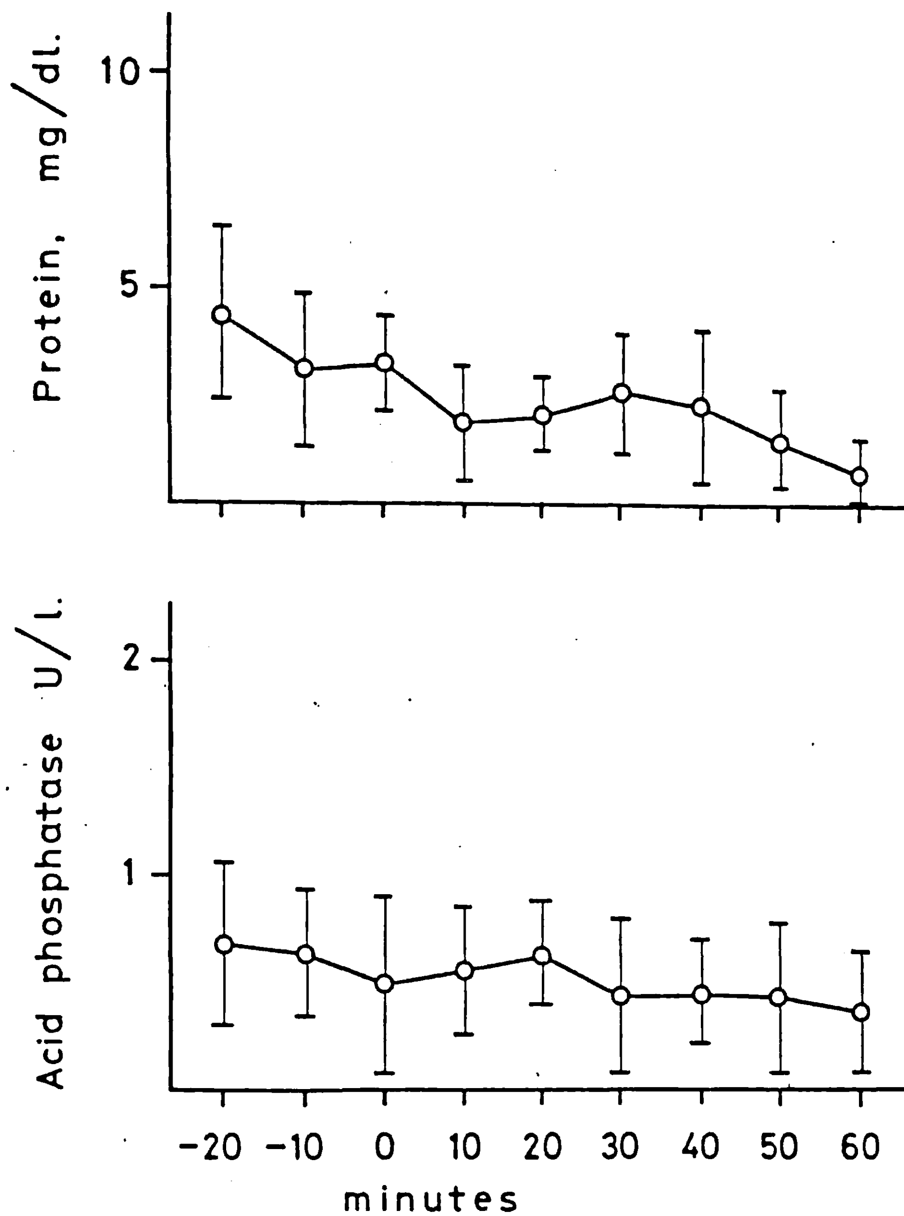


Fig 3:13 Effect of secretin (100 µg) on perfusate protein and acid phosphatase levels. Secretin did not cause any significant elevation in either parameter following its infusion. Mean ± SD, n = 4.

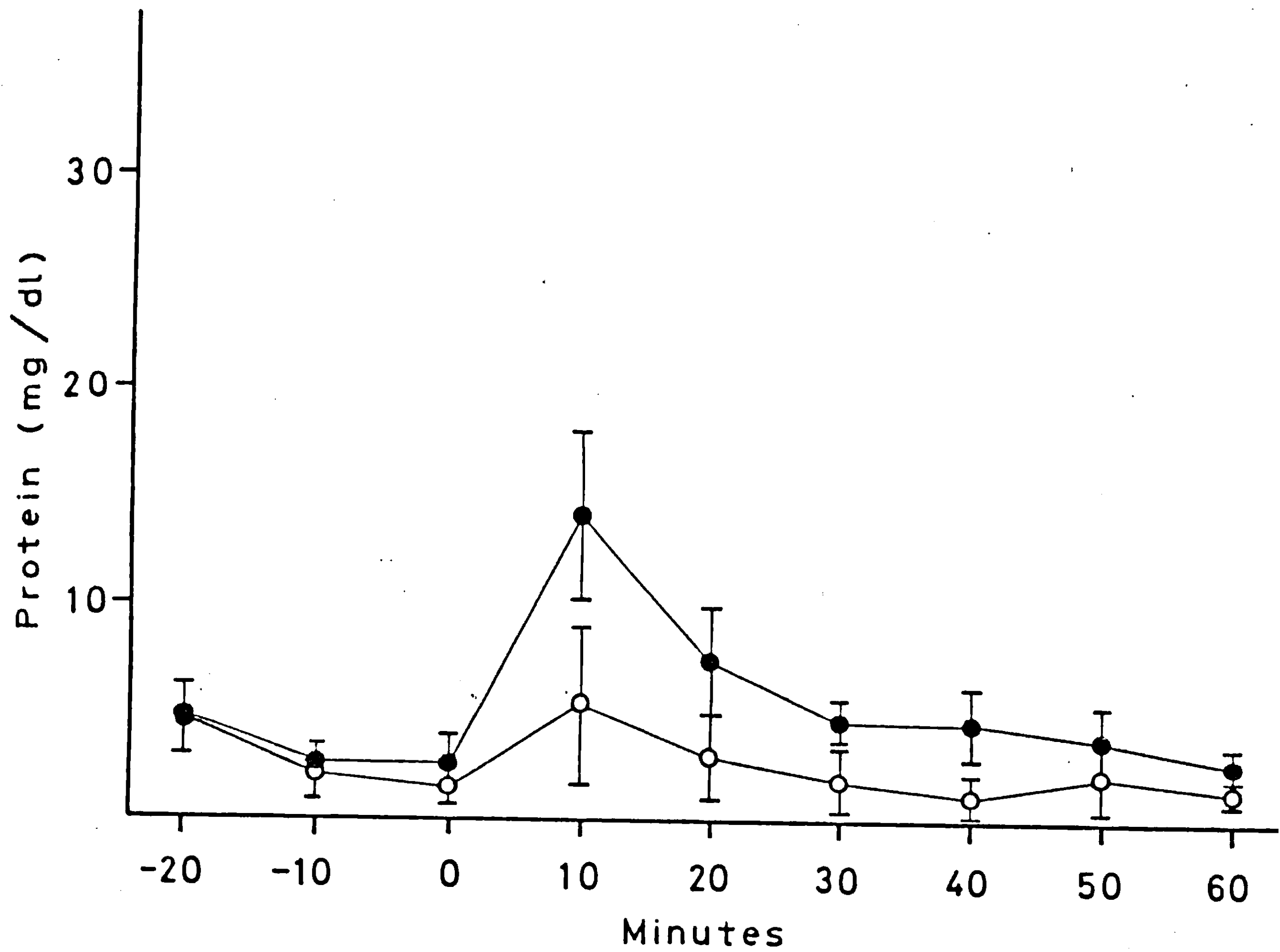


Fig 3:14 Effect of 5 mM Bt_2c-GMP on the response produced by infusion of 10 μg of CCK-33. Infusion of CCK-33 alone (●, n = 4) produced a significantly larger response than the infusion of 10 μg CCK plus 5 mM Bt_2c-GMP (O, n = 3). Mean ± SD.

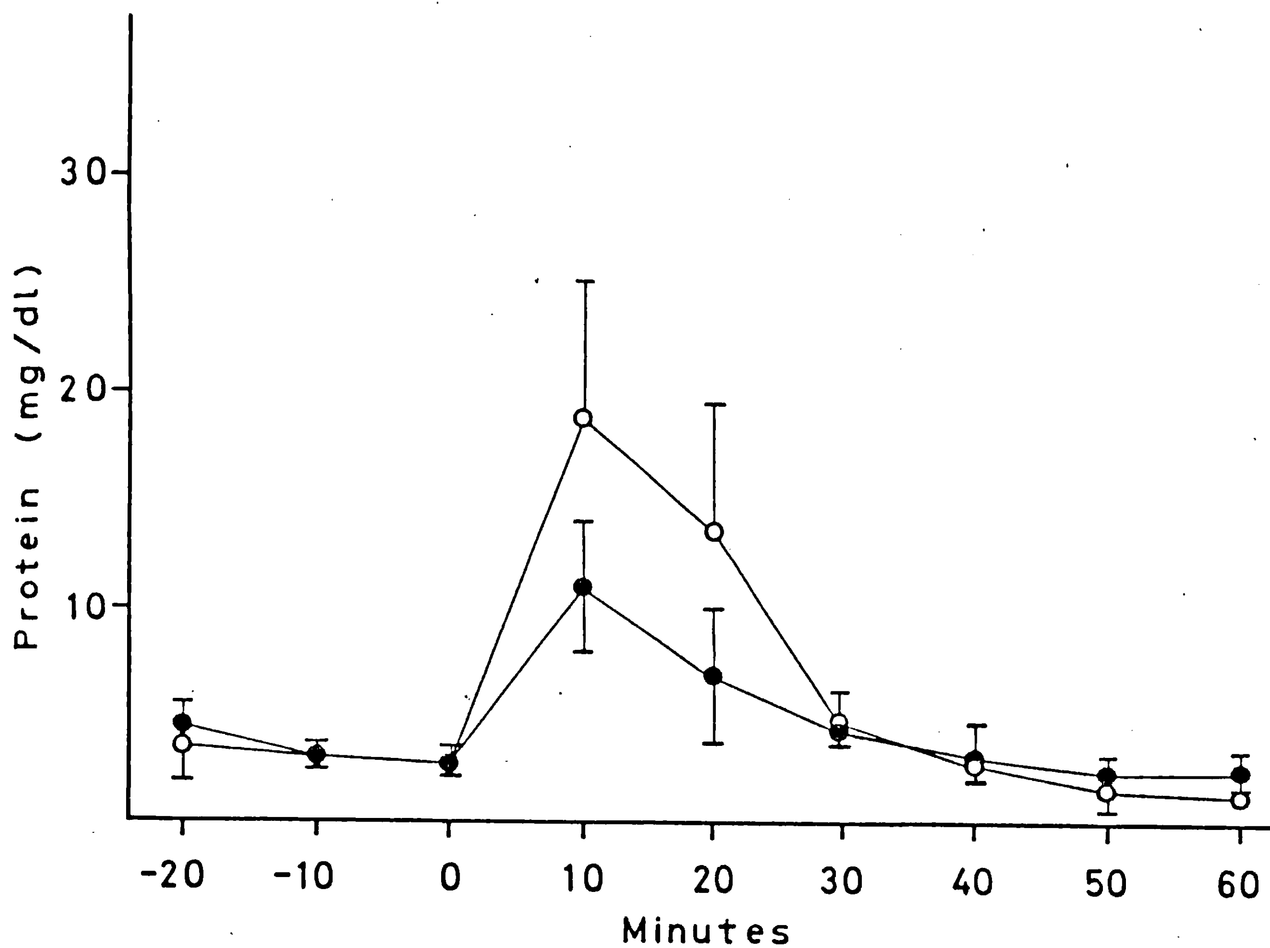


Fig 3:15 Effect of 5 mM Bt_2c-GMP on the response produced by infusion of 100 µg CCK-8 (sulphated). Infusion of sulphated CCK-8 alone (O, n = 3) produced a significantly larger response than the infusion of 100 µg sulphated CCK-8 plus 5 mM Bt_2c-GMP (●, n = 4). Mean ± SD.

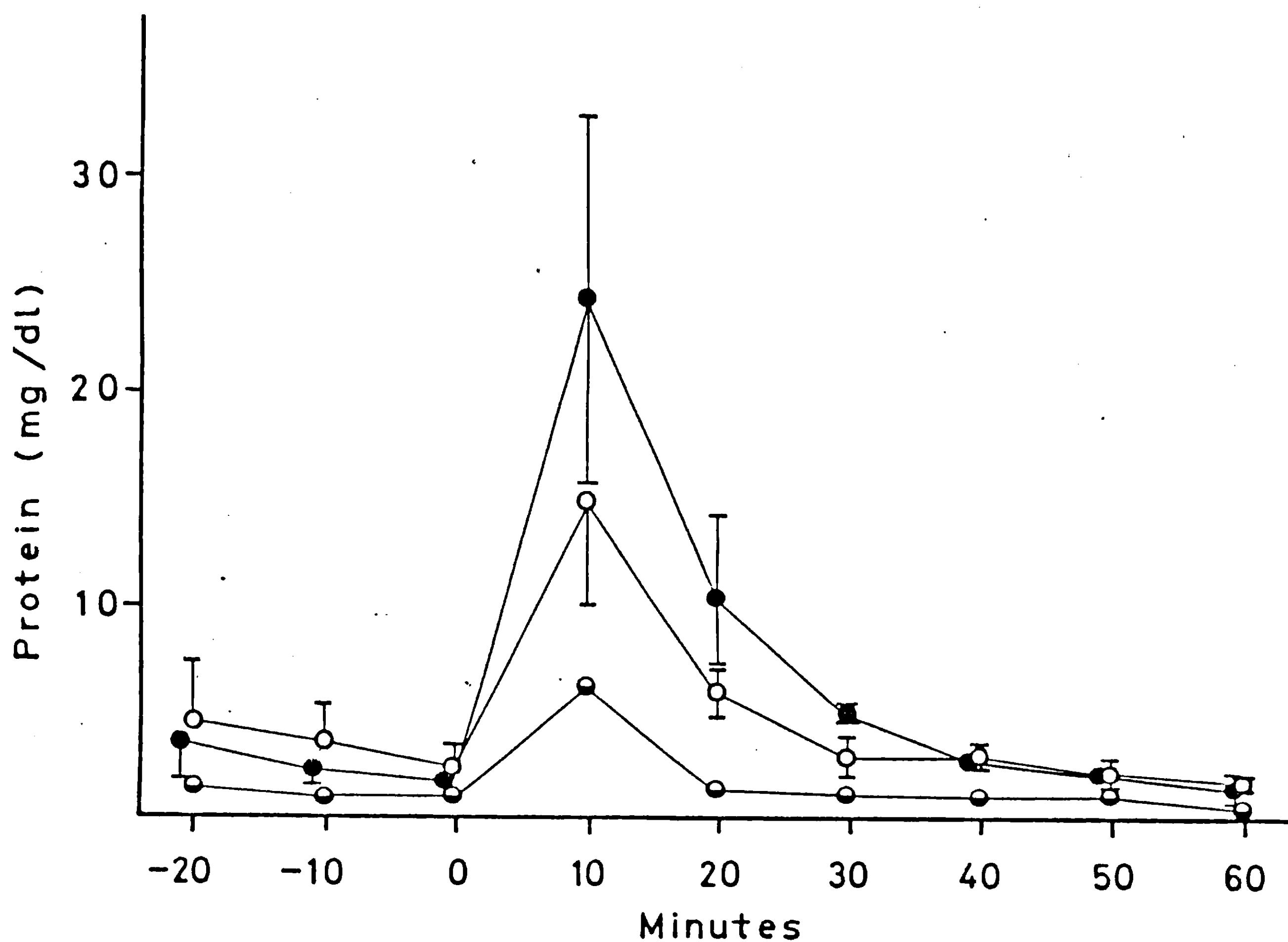


Fig 3:16 Effect of bombesin 6 - 14 nonapeptide on perfusate protein levels following infusion of 0.1 μg (◐, n = 2), 1.0 μg (O, n = 3) and 10 μg (●, n = 4). All doses increased perfusate protein levels. Mean ± SD.

quarter of the 100 µg (8.76-9.41x10^{-8} moles) CCK-8 doses (Fig 3:11), produced a far greater response than that which was observed with the octapeptide forms. A dose of 10 µg (2.55x10^{-9} moles) produced a maximal response of 13.9 ± 4.0 mg/dl after 10 mins, similar to the effect of 100 µg (8.76x 10^{-8} moles) of CCK-8.

The infusion of 100 µg (3.27x10^{-8} moles) of secretin (Fig 3:13) produced no significant elevation of either protein or acid phosphatase levels. The response following secretin infusion was not significantly different to the infusion of vehicle Ringer alone (Fig 3:6).

Effect of Bt2c-GMP on the response to CCK

The addition of 5 mM of Bt2c-GMP to each dose of peptide had a significant influence on the response produced by infusion of CCK-8 and CCK-33 alone. Repeated infusions of 200 µl of ringer containing only 5 mM of Bt2c-GMP produced no significant response.

Whereas 10 µg (2.55x10^{-9} moles) of CCK-33 caused a maximum perfusate protein level of 13.9 ± 4.0 mg/dl, the same dose with 5 mM Bt2c-GMP added produced a maximum level of only 5.3 ± 3.7 mg/dl (Fig 3:14).

Similarly a 100 µg (8.76x10^{-8} moles) dose of sulphated CCK-8 produced a maximum response of 18.9 ± 6.3 mg/dl, but if infused with 5 mM Bt2c-GMP this response was almost halved, the maximum level of perfusate protein being 11.0 ± 2.9 mg/dl (Fig 3:15).

Effect of bombesin

Bombesin 6-14 nonapeptide was the most potent secretagogue tested. Doses of 0.5, 5.0 and 10 µg (9.33x10^{-11}, 9.33x10^{-10} and 9.33x 10^{-9} moles) produced maximum levels 10 mins post-infusion, of 6.1, 14.9 ± 4.6 and 24.4 ± 8.7 mg/dl respectively (Fig 3:16). The time course of

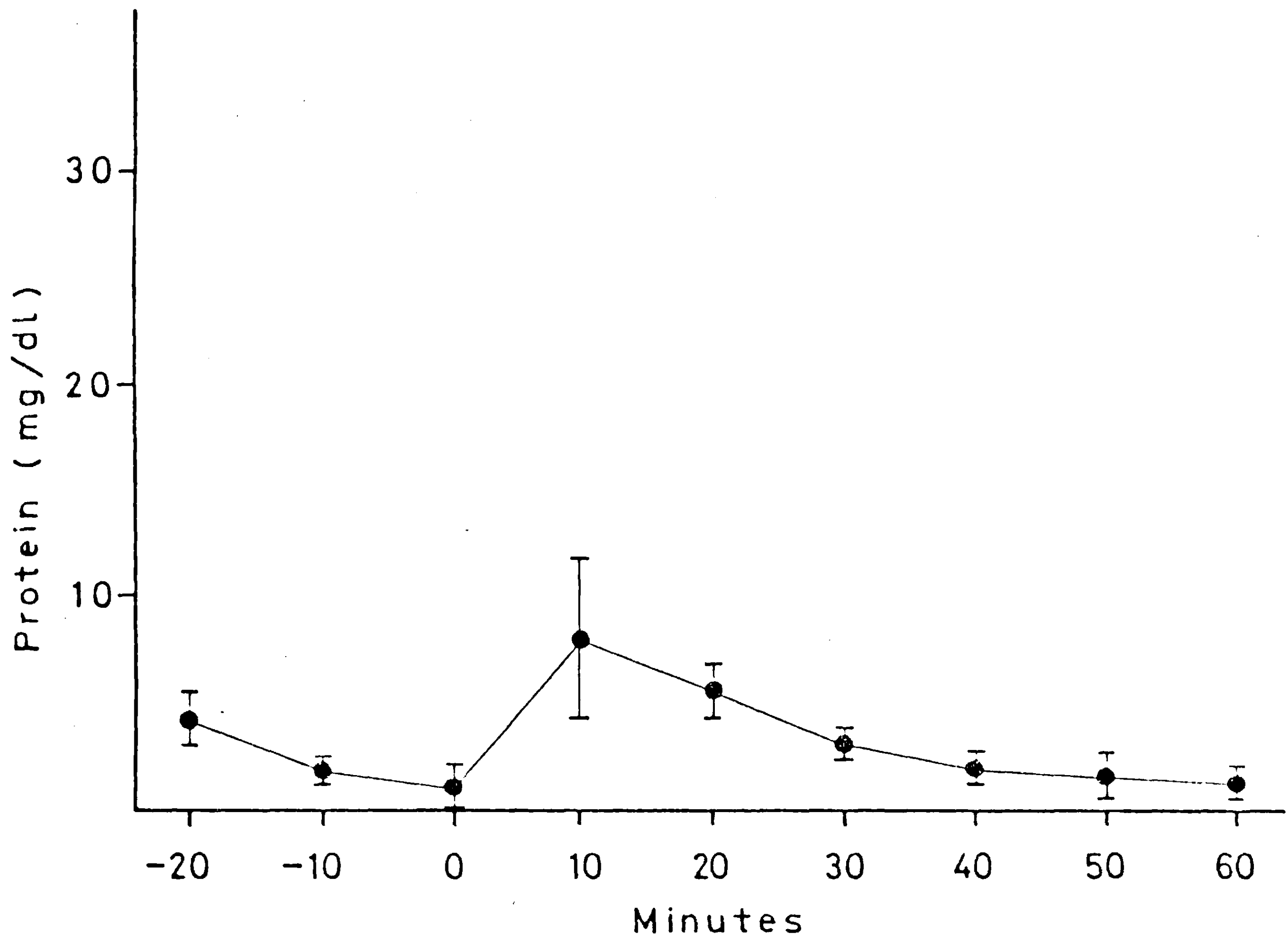


Fig 3:17 Effect of an infusion of 100 μg of caerulein on perfusate protein levels. Caerulein produced a small but significant elevation of perfusate protein lev ls. Mean ± SD, n = 3.

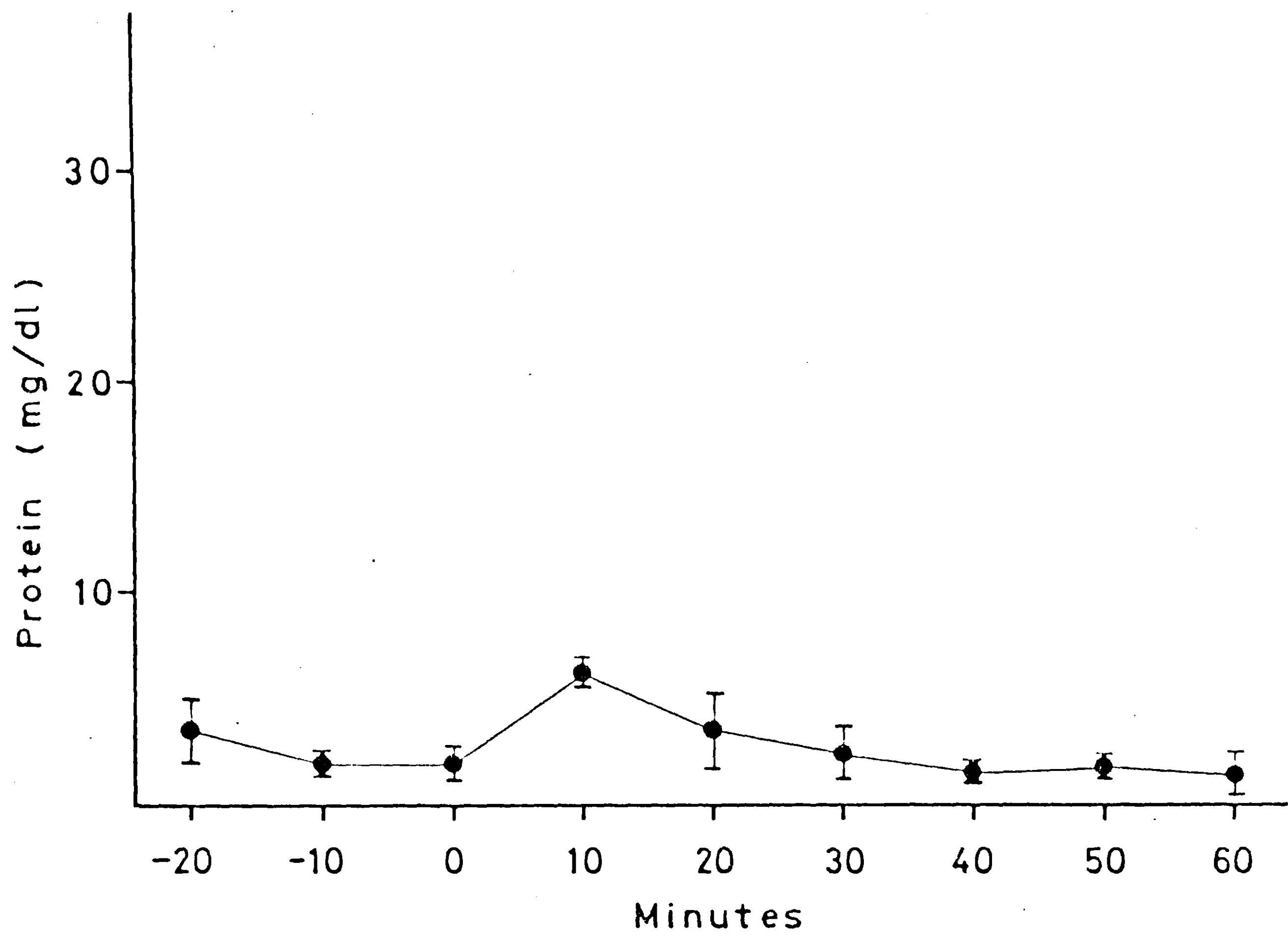


Fig 3:18 Effect of an infusion of 100 μg of physalaemin on perfusate protein levels. Physalaemin produced a significant elevation of perfusate protein levels. Mean ± SD, n = 4.

the response was superficially identical to that observed following CCK infusion (ie Fig 3:10) but the potency of the peptide was about an order of magnitude greater.

Effect of caerulein

A 100 µg (7.40×10^{-8} moles dose of caerulein produced a small but highly significant response (Fig 3:17). The maximum response was measured 10 mins post-infusion (8.1 ± 3.7 mg/dl) and the level was still significantly elevated during the subsequent 10 minute period (5.5 ± 1.1 mg/dl).

Effect of physalaemin

Physalaemin was observed to have a similar potency to caerulein (Fig 3:18). A dose of 100 µg (7.90×10^{-8} moles elicited a significant response with a maximum level being recorded 10 mins post-infusion, of 13.3 ± 9.0 mg/dl.

Effect of glucagon

Glucagon, like secretin (Fig 3:13), produced no significant effect on perfusate protein levels. Mean perfusate levels remained substantially unaltered by a 100 µg (2.82×10^{-8} moles infusion of glucagon, the pre-perfusion protein levels being 1.6 ± 0.6 mg/dl, and those 10 mins post-perfusion being 1.8 ± 0.8 mg/dl.

DISCUSSION

The results presented in this chapter indicate that a number of regulatory peptides stimulate the release of a proteinaceous secretion into the gastric lumen of Styela clava. In the experiments where it was measured, acid phosphatase showed a simultaneous and parallel

elevation, which confirms that this material may be enzymatic in nature. Subsequent attempts to further identify the nature of the secretion by attempting to measure amylase and protease activity in the perfusate were unsuccessful and so this may indicate their absence. In this context it is perhaps significant that Thomas (1970) was unable to demonstrate the presence of either protease or lipase activity by histological methods in zymogen granules in the gut of Ciona. For the sake of simplicity this material, detectable by the Lowry method will be considered to be the product of the zymogenic protein secreting cells, as there appears to be no other histological cell type capable of releasing a proteinaceous secretion (see chapter 1). It is hard to imagine that this material should turn out not to be involved in extracellular digestion, but it is readily conceded that the material has not been adequately defined. As has been noted, extracts of the ascidian gut have been found to contain many enzymes and it seems inconceivable that in future studies the proteinaceous perfusate, induced by the secretagogues mentioned in this chapter, will not be shown to contain enzymes in addition to acid phosphatase.

Acid phosphatase was measured in initial studies because of its presence in both vertebrate pancreatic acinar cells (Munger, 1973; Wachstein and Meisel, 1959) in the 'pancreatic' protein synthesising cells of the digestive caecum of Branchiostoma (Welsch, 1975; Welsch and Storch, 1969) and in the zymogen cells of Styela (Relini Orsi, 1968) where it is located in the apical cytoplasm at the site of zymogen granule release. It was therefore felt that its presence might be taken as indicative of zymogen granule release. The assay was available in kit form and was thus simple to use but having established that it was detectable in parallel with the Lowry protein, the considerable cost of performing this test meant that in later

experiments it was not determined.

From the results obtained in this study it is clear that whilst all the secretagogues which effect PI, intracellular calcium and c-GMP in the vertebrate pancreatic acinar cell, increase the levels of perfusate protein, glucagon and porcine secretin have no effect. Given that secretin-like immunofluorescence is readily identifiable in Styela gastric epithelium, whereas CCK-like immunofluorescence was not observable, this observation was unexpected.

Although much of the work on pancreatic acinar cell receptors has been perfomed in the guinea pig, there is evidence that these cells are atypical in having well developed receptors to VIP/secretin, which have no secretagogue action in many other vertebrate species. In vivo, in most mammalian species tested, secretin has a purely hydrelatic action, with no concomitant ecbolic effect (see Introduction, chapter 2) and it is therefore reasonable to suppose that in these other species the pancreatic acinar cells do not possess secretin receptors. It is however probable that the c-AMP dependent second messenger system is still operative, as c-AMP and its derivatives have a secretagogue action in these cells in the rabbit (Ridderstap and Bonting, 1969) and mouse (Kulka and Sternlicht, 1968).

Grossman and Ivy (1946) first suggested that secretin acted on the duct cells of the exocrine pancreas, whilst CCK acted on the acinar

cells. Despite the small amount of overlap that exists, this basic premise still appears to be substantially correct. This premise has received considerable support from the micropuncture studies by Schulz et al (1969), where they measured the composition of samples of pancreatic juice following administration of CCK and secretin.

It is also attractive to speculate that perhaps the c-AMP second messenger system is not present in these ascidian pre-pancreatic zymogen cells, although it must be stressed that the nature of the second messenger has not been directly examined. The effect of secretin and also VIP and glucagon, on enzyme secretion in the pacific hagfish (Eptatretus stouti) has been tested. Hagfish, like Styela, has a number of zymogen cells homologous with pancreatic acinar cells distributed through the intestinal epithelium (Barrington, 1972). Whilst CCK-33 stimulates lipase secretion, secretin, VIP and glucagon have no effect (Vigna & Gorbman, 1979). This is entirely consistent with the current observations in Styela.

If the secretin-like immunofluoresence indicates a secretin-like peptide product, which is not involved in enzyme secretion, then the question of its role remains unanswered. Recently extracts of Styela have been prepared at the Karolinska Institute and in London, in collaboration with Prof Mutt, which have a secretin-like action in cat and rat bioassays (Thorndyke and Bevis, 1983). The speculative suggestion has been made that the action of this peptide in the ascidian is on mucous cell secretion (Thorndyke and Bevis, 1984). This hypothesis is attractive, due to the physical presence of the secretin containing cells scattered amongst the ciliated mucous cells in the mucous caps (Thorndyke, 1977), in an ideal position to control their output by a paracrine mechanism. Also, although secretin was originally thought only to stimulate pancreatic secretion and hepatic

bile secretion (Bayliss and Starling, 1902a) the list of its actions has grown considerably over the years and it has now been shown to play a role in the stimulation of gastric mucus secretion (Andre et al, 1972; Kaura et al, 1980; Kowalewski et al, 1978; Vagne et al, 1982). The majority of assays performed were however on the CCK group of peptides and the results here were unexpected.

Studies of CCK analogues have made considerable reference to the importance of the sulphation of the tyrosine residue at position 7 from the C terminus for full potency (Chowdhury et al, 1976; Jensen et al, 1980; Kaminski et al, 1977; Ondetti et al, 1970b). Desulphation or translocation from position 7 to 6, as in gastrin, considerably reduces the activity both for gall bladder contraction and in the acinar cell system (Gardner and Jensen, 1983b).

It has however been suggested that the cholecystokinetic action of CCK is more reliant on the sulphation of the tyrosine than is the pancreozyminic activity (Mutt, 1982a). This claim is supported by the comparative study carried out by Dockray (1973) in which CCK-8 was about 4000 times more potent than pentagastrin as a stimulant of pancreatic fluid secretion but only 2,500 times more potent than pentagastrin as a stimulant of protein secretion. This situation also occurs in the case of caerulein, where sulphation is less important for the pancreozyminic than the cholecystokinetic activity (Johnson et al, 1970). It is arguable that this reflects the situation in Styela where it appears that sulphation of the tyrosine is unnecessary.

If this were the case then one might expect that the situation in lower vertebrates might reflect an intermediate situation. This does not seem to be the case with regard to studies on the cholecystokinetic action. Vigna and Gorbman (1977) have shown that in the coho salmon

(*Oncorhynchus kisutch*) sulphated gastrin and CCK are roughly equipotent in causing in vitro gall bladder contraction but their unsulphated counterparts are about 1000 times less active.

Unfortunately no studies have been performed on the effect of sulphation of the tyrosine on CCK/caerulein/gastrin mediated enzyme secretion in the gut or pancreatic homologues. Vigna and Gorbman (1979) have studied the effects of glucagon, VIP, secretin and CCK-33 on gut enzyme (lipase) secretion from the primitive pre-pancreatic zymogen cells in the intestine of the pacific hagfish, *Eptatretus stouti*. In the same study they also tested the effect of gastrins-17-I and II (G-17 non-sulphated and G-17 sulphated) on gall bladder contraction, neither of them (nor CCK) being active. However they did not examine their effect on lipase secretion. Thus the question of whether or not other primitive pre-pancreatic systems are able to distinguish between sulphated and non-sulphated forms apparently remains unresolved.

Another unexpected finding was that CCK-8 is not more potent than CCK-33 in the *Styela* perfusion system. Following the original isolation of CCK, the octapeptide was reported to be about 2.5 times more potent on a molar basis than CCK-33 (Jorpes, 1968; Ondetti et al, 1970; Rubin et al, 1969).

Evidence recently presented by Lamers et al (1983) and Solomon et al (1984) suggests that these early results were misleading, because of the differential adsorption of the peptides to glass and plastic, a problem which was not then recognised. Lamers et al find that CCK-8 and CCK-33 are equipotent on a molar basis, which supports an earlier observation by Jensen et al (1981) that shows CCK-8, CCK-33 and CCK-39 to be equipotent in their ability to stimulate enzyme secretion from

the isolated porcine pancreas.

The response to the two CCK variants in Styela is however not equal in molar terms, but the larger form, CCK-33, is between 4 and 40 times more potent. This would seem to be indicative of a fundamental requirement of more than the octapeptide sequence for full activity.

Straus et al (1978) have described an enzyme from porcine and canine brain with trypsin-like properties (ie cleavage of lysyl and arginyl peptide bonds) which appears to form CCK-12 and CCK-8 from CCK-33. Certainly limited degradation with trypsin of all larger forms of CCK produces CCK-12 in vitro which with more vigorous trypsin treatment can be converted to CCK-8 (Mutt, 1979). It is possible that a similar situation could be obtained in Styela, possibly with a more specific enzyme cleaving only the arg-ile bond, thus producing CCK-12. Preliminary observations, using gel filtration and region specific antisera suggest that a CCK-like peptide of about this size could be present in extracts of Styela gut (Thorndyke and Bevis, 1983).

Also surprising was the observation that caerulein is only a comparatively weak secretagogue in Styela. A 100 μg infusion of caerulein evokes approximately the same response as 10 μg of CCK-8. This indicates that although the sulphation of the tyrosine at position 7 is of no significance, the substitution of the methionine for threonine at position 6 reduces the potency ten-fold.

This result was unexpected as in vertebrate systems caerulein has been reported to be more potent (Jorpes, 1968) or at least similar in potency (Gardner, 1979b; Solomon et al, 1984) to CCK-8, which is not surprising considering how similar it is in structure (Anastasi et al, 1968).

It remains uncertain whether caerulein is truely phylogenetically old, or merely an evolutionary cul-de-sac, found in a group of amphibians which are highly specialised and a long way from the direct evolutionary descent of the higher vertebrates (Romer, 1971). Larsson and Rehfeld (1977) have suggested that it might represent the parent molecule from which both CCK and gastrin subsequently arose. However the evidence which they present is not incompatible with a more CCK-like precursor. Radioimmunoassays of Styela gut extracts (Thorndyke & Bevis, 1983) have revealed the evidence of a peptide similar in size to caerulein but more CCK-like in its antigenic behaviour.

The sharp discrimination between CCK-8 and caerulein observed in the present study suggests that caerulein is not present in these animals or that if it is, it plays some other role in their physiology. Although caerulein has been reported once in a sub-amphibian group (Van Noorden and Pearse, 1974), this was with an antiserum which readily cross-reacted with CCK and gastrin. Caerulein has not been demonstrated in the gastrointestinal tissue of any other vertebrate species (Mutt, 1982b) or to have any biological effect in any sub-amphibian species. It is therefore possible that caerulein may not be a phylogenetically old peptide, but a recent invention of the Amphibia.

Another peptide, synthetic bombesin 6-14 nonapeptide, originally isolated as a tetradecapeptide from amphibian skin (Anastasi et al, 1971) was the most potent secretagogue tested in this assay. Bombesin was found to be about ten times more potent than CCK-8.

Bombesin is closely related to gastrin releasing peptide (GRP) (McDonald et al, 1978; 1979; 1980), indeed the nonapeptide, used in this study, differs from porcine GRP nonapeptide by only one amino acid

residue at position 7, which is Gln in bombesin but His in GRP.

Both bombesin and GRP are potent stimulators of gastric acid secretion. This effect is brought about by their release of gastrin from the G cells of the antral mucosa (Bertaccini et al, 1973; 1974; Erspamer, 1980). Bombesin is also known to have a direct secretagogue action on the pancreatic acinar cell (Jensen et al, 1978a; 1978b) although its in vivo action may be due in part to its ability to induce the release of CCK, which also acts directly on the acinar cell (Erspamer et al, 1974).

Bombesin is also known to release a number of other regulatory peptides. These include enteroglucagon, motilin, neurotensin (Bloom et al, 1979), gastric inhibitory peptide (Becker et al, 1978) and pancreatic polypeptide (Taylor et al, 1978).

In view of this wide range of releasing effects and in particular the CCK and gastrin releasing effects, it is tempting to speculate that the abnormally high potency of bombesin nonapeptide in Styela may be due, at least in part, to a release of an endogenous secretagogue from the endocrine-like cells in the gastric epithelium.

Another amphibian-skin peptide, physalaemin (Erspamer et al, 1977) a substance P related peptide, also acts as a secretagogue in Styela. At a comparable dose, by weight, physalaemin is more potent than caerulein.

Specific physalaemin receptors, which act by increasing PI turnover, have been demonstrated to form part of the rich receptor complement of guinea pig acinar cells (Gardner and Jensen, 1980). That physalaemin appears more potent than caerulein in Styela, indicates that it is not acting on the CCK receptor but on a physalaemin

receptor. The low potency of caerulein compared to CCK demonstrates the specificity of the CCK receptor. It is therefore unlikely that the action of physalaemin could be mediated through this specific CCK receptor as physalaemin is not CCK-like. It is not however possible to say anything about this physalaemin receptor and its specificity.

One other peptide, glucagon (which structurally belongs to the VIP/secretin/PHI group of peptides) was found to have no effect on enzyme secretion in Styela. This is perhaps not unexpected due to its structural similarity to secretin which has no secretagogue effect in Styela and because glucagon is not a pancreatic secretagogue in vertebrates (Deschodt-Lanckman et al, 1975).

Glucagon has been described as a secretagogue in mouse pancreatic acinar cells (Manabe and Steer, 1979; Singh, 1980a; 1980b) but this has been subsequently ascribed to an unknown contaminant in the natural glucagon preparations which had been used in the previous studies (Pandol et al, 1983).

Brief mention was made in the introduction to this chapter of specific receptor antagonists which have been studied in vertebrates, particularly the guinea pig, pancreatic acinar cells. With respect to the CCK receptor, three classes of antagonist have now been described.

The first class was described by Peikin et al (1979) who reported that butyryl derivatives of c-GMP, specifically Bt2c-GMP, are able to specifically inhibit the action of CCK on the pancreatic acinar cell. It is now known that CCK receptors in other tissues are also specifically inhibited by Bt2c-GMP (Davison and Najafi-Farashah, 1981; 1982a; Hutchison and Dockray, 1980; Kasbekar et al, 1983; Poitras et al, 1980).

The mechanism by which Bt2c-GMP acts is uncertain. It has been suggested that it does not act on the CCK receptor so as to block its activation by CCK but that the nucleotide combines with CCK so as to interfere with its ability to bind to its receptors (Miller et al, 1983). Gardner (1983) has however criticised this study and has disagreed with their conclusions.

A second class of CCK receptor antagonists, consisting of amino acid derivatives, was reported by Hahne et al (1981). Proglumide, a glutaramic acid derivative, and benzotript, an N-acyl derivative of tryptophan, are specific CCK antagonists in pancreatic acini as well as gall bladder, ileal (Davison and Najafi-Farashah, 1982b), and gastric muscle (Collins and Gardner, 1982). Other N-acyl derivatives of L-tryptophan have also been reported to inhibit CCK-stimulated amylase secretion (Jensen et al, 1983b).

A third class of antagonist consisting of COOH-terminal fragments of CCK, has also been described (Jensen et al, 1983a).

In the present study we found that addition of 5 mM Bt2c-GMP to CCK-33 and CCK-8 caused a highly significant reduction in the observed response. This is consistent with its effect on the CCK response in vertebrate systems. However the present doubt over the mode of action of Bt2c-GMP makes its unsafe to conclude that this is necessarily evidence for structural similarity between the ascidian and vertebrate CCK receptor.

It would perhaps be expected that the ascidian CCK receptor might be similar to its vertebrate counterpart. It has been reported that the insulin receptor is functionally more conserved in a range of vertebrates from cyclostome to human than is insulin (Muggeo et al, 1979a; 1979b). The present results however argue for a receptor with

some structural differences, as has been pointed out earlier in this chapter.

It is interesting that preliminary observations using this assay system have shown that infusion of only a few hundred nanograms of relatively impure acid extracts of Styela gut can evoke a response comparable to many micrograms of pure CCK (Thorndyke & Bevis, 1983). This discrepancy between the low potencies of sulphated and non-sulphated CCKs and the higher potency of impure extracts, argues against the receptor involved being the same as the CCK-receptor. Possibly the response observed with the CCKs represents only a weak interaction with either a primitive CCK receptor, or a receptor to an unknown hormone, which could perhaps be purified from Styela gut extracts, using this Styela perfusion system to follow the degree of purification.

None of the peptides described in this chapter have been used at 'physiological' concentrations. This may indicate that there is only very weak hormone/receptor interactions or it may reflect a limitation of the method; ie the heart is the only vessel large and strong enough to allow cannulation and therefore the already sluggish circulation must perforce be halted. It is therefore possible that the concentration of peptide at the receptor is much lower. Alternatively, if the secretion truely reflects a 'paracrine' release from adjacent cells a comparatively high molar concentration of peptide might occur locally, before being dispersed in the general circulation. Therefore to achieve that high concentration at the receptor, a non-physiological quantity of peptide would need to be infused into the general circulation.

The action of regulatory peptides in vertebrate systems can be

divided into three modes of action (Krieger, 1983). The endocrine role is the original mechanism proposed by Bayliss and Starling in 1902. Peptides are released into the general circulation in response to some physiological stimulus and from there they provoke physically distant target cells to respond. By infusion of exogenous peptides at physiological concentrations characteristic biological activities can be observed (Grossman, 1977).

The neurocrine role of action pertains to the numerous peptide containing nerves of the gastrointesintal tract, for example somatostatin, CCK, VIP and substance P. Although the physiological role of these peptides is in most cases unresolved it is probable that they function as neurotransmitters or neuromodulators. However the neural products may in some cases be spread over a wider area, or even be released into the bloodstream as neurohormones. This type of action is well recognised in the control of the pituitary gland but it has also been proposed as a mode of VIP action (Fahrenkrug, 1979).

In the paracrine mode of action hormones are released into the local tissue fluid within an organ, to act purely as local hormones. This function is still hypothetical as it is difficult to examine experimentally. In the case of somatostatin a paracrine mechanism is argued for on structural grounds (Alumets et al, 1979; Kusumoto et al, 1979; Larsson et al, 1979) as long processes extend from antral somatostatin cells to adjacent cells including G-cells.

One final question remains unresolved. Although secretin-like immunoreactivity has been clearly observed in the gastric epithelium, CCK-like immunoreactivity has proved impossible to localise by three different antisera which would react with mammalian CCK-33. The sensitivity of the gut to peptide secretagogues implies their presence

and a CCK-like peptide has indeed been identified in extracts of Styela gut (Thorndyke + Bevis, 1983).

Weinstein (1972) has suggested on theoretical grounds that both the secretin/glucagon/VIP family and the gastrin/CCK family arose from a single parent molecule which he termed prosecgastrin. Holmquist et al (1979) have suggested that in lampreys small CCK/gastrin- and secretin/glucagon-like peptides may already be produced separately, while Van Noorden and Pearse (1974; 1976) have demonstrated that a single endocrine cell type found in the gut of lamprey and the more primitive Branchiostoma, has the ability to react with both anti-glucagon and anti-gastrin sera. They suggest that this double immunoreactivity may be due to the presence of a single prosecgastrin type of prohormone. It is possible that this situation occurs in Styela.

It is well known that many small bioactive peptides are cleaved from larger precursor proteins that are processed during their migration through the cell into storage or transport vesicles to produce the active products. This proteolytic processing occurs after Golgi associated post-translational modification such as glycosylation, phosphorylation and sulphation have occurred. Proteolysis normally occurs at paired basic residues, suggesting that a small number of specialised tryptic proteases exist with great site selectivity but which can process many sites within the precursor.

Many prohormones contain a single active peptide although the 'packaging' sequence, as in the case of insulin c-peptide may be co-secreted. However in 1977 the first of the multifunctional or

polyprotein type of hormone precursor, proopiomelanocortin, was described (Mains et al, 1977).

Molecular biology and the techniques of gene cloning have provided an avalanche of hormone precursors, many of which contain more than one regulatory peptide within their structure. Examples include proenkephalin (Comb et al, 1982), provasopressin/neurophysin (Land et al, 1982), prooxytocin/neurophysin (Land et al, 1983) and preprovasoactive intestinal peptide/PHM-27 (Itoh et al, 1983).

In view of this plethora of polyprotein type prohormones it does not seem unreasonable to postulate that the cap cells may contain a CCK-like product in addition to the secretin-like product. However, for some reason it is not possible to visualise it by immunocytochemical means.

If the CCK-like peptide is not located in the cap cells, then it is possible that they exist in some other E-L cell type. It is not impossible for example, that it is a product of the type II oesophageal cells, in addition to their insulin-like product. It is also possible although most improbable that they are not sited in the gastrointestinal mucosa. In this case it is hard to see how they could respond to the presence of food stimuli in the gut.

E-L cells are however present elsewhere. E-L cells, which immunoreact with calcitonin antiserum, have been described adjacent to the iodine-binding thyroidal cells of zone 7 of the endostyle of Styela (Thorndyke and Probert, 1979). Fritsch et al, (1980) have demonstrated cells which immunostain with antisera to substance P, neurotensin and bombesin in the pharyngeal epithelium of Ciona intestinalis. This epithelium has not been examined for E-L cells in Styela.

Neurosecretory cells have also been described in the ascidian neural ganglion (Dawson and Hisaw, 1964) and bombesin-, secretin-, VIP-, somatostatin-, substance P-, calcitonin-, CCK/gastrin-, met-enkephalin-, ACTH- and prolactin-like immunoreactivity have also been demonstrated within the neural ganglion of a range of ascidian species (Fritsch et al, 1979; 1982; Georges and Dubois, 1979; 1984; Pestarino, 1983; 1984; Pestarino and Tagliafierro, 1982; Thorndyke, 1981; 1982). But considering the physical remoteness of these sites from the gut and the primitive cardio-vascular system, coupled with the lack of evidence for direct innervation of the gut of _Styela_, it is improbable that they are producing peptides which have direct control over gastrointestinal functions.

APPENDIX 1

BUFFERS

Cacodylate buffer

Stock A: 0.2 M sodium cacodylate

4.28 g sodium cacodylate (mol wt 214) in 100 ml distilled water

Stock B: 0.2 M HCl

1.7 ml hydrochloric acid in 100 ml distilled water

pH 7.2 buffer: add 25 ml of A to 2.1 ml of B and make up to 100 ml with distilled water

Phosphate buffer

Stock A: 0.2 M sodium dihydrogen orthophosphate

3.12 g sodium dihydrogen orthophosphate (mol wt 156) in 100 ml distilled water

Stock B: 0.2 M disodium hydrogen orthophosphate

2.83 g disodium hydrogen orthophosphate (mol wt 142) in 100 ml distilled water

pH 7.0 buffer: add 19.5 ml of A to 30.5 ml of B and make up to 100 ml with distilled water

pH 7.4 buffer: add 9.5 ml of A to 40.5 ml of B and make up to 100 ml with distilled water

Tunicate Ringer

Devised by Thorndyke from data for the ionic composition of ascidian body fluids (Robertson, 1954).

	g/l
Sodium chloride	25.90
Potassium chloride	0.75
Calcium chloride	1.05
Magnesium chloride	5.295
Sodium sulphate	1.63
Sodium bicarbonate	0.06
Glucose	1.00

Acetate buffer

Stock A: 0.2 M acetic acid

1.2 ml glacial acetic acid in 100 ml distilled water

Stock B: 0.2 M sodium acetate

1.64 g sodium acetate anhydrous (mol wt 82) in 100 ml distilled water

Add x ml of A to y ml of B. Then make up to 100 ml with distilled water.

pH	x ml A	y ml B
3.8	44.0	6.0
4.0	41.0	9.0
4.2	36.8	13.2
4.4	30.5	19.5
4.6	25.5	24.5
4.8	20.0	30.0
5.0	14.8	35.2
5.2	10.5	39.5

Tris-HCL buffer

Stock A: 0.2 M Tris

2.4 g Tris (hydroxymethyl) amino methane (mol wt 121) in 100 ml distilled water

Stock B: 0.2 M HCL

1.7 ml hydrochloric acid in 100 ml distilled water

pH 7.2 buffer: add 25 ml of A to 22.1 ml of B and make up to 100 ml with distilled water

pH 7.6 buffer: add 25 ml of A to 19.2 ml of B and make up to 100 ml with distilled water

Phosphate buffered saline

Sodium chloride 17g

Disodium hydrogen orthophosphate 2.56g

Sodium dihydrogen orthophosphate 0.312 g

Distilled water 2 l

FIXATIVES

Bouin

Saturated aqueous picric acid 75 ml

Formaldehyde (40%) 25 ml

Glacial acetic acid 1 ml

Will keep indefinitely if glacial acetic acid is not added until just before use. Seawater Bouin may be prepared by using saturated seawater picric acid

6% Glutaraldehyde

0.2 M sodium dihydrogen orthophosphate 48.75 ml

(3.12 g in 100 ml distilled water)

0.2 M disodium hydrogen orthophosphate 76.25 ml

(2.83 g in 100 ml distilled water)

25% glutaraldehyde

Check that pH is 7.0, add more disodium hydrogen orthophosphate if necessary. Then make up to 250 ml with distilled water and store in a stoppered glass vessel at 4°C.

Marine fixative

25% glutaraldehyde 24 ml

0.2 M sodium cacodylate (made with seawater) 100 ml

Distilled water 51 ml

Filtered seawater 25 ml

This mixture should produce a solution of 1000 milliosmoles.

Adjust pH to 7.2 using 0.2 M HCl

STAINS

Reynold's lead citrate (Reynolds, 1963)

Add 1.33 g of lead nitrate and 1.76 g of sodium citrate to 30 ml of distilled water. Shake vigorously for the first minute and each 5 mins thereafter over a 30 min period. A heavy white precipitate of lead citrate is formed and this agitation facilitates formation. Add 8 ml of freshly made 1 M sodium hydroxide (carbonate free) whilst stirring and dilute to 50 ml with distilled water. At this point the lead citrate precipitate dissolves. The stain is now ready for use, and should be stored at 4°C in a stoppered bottle. With age a white precipitate forms due to the absorption of atmospheric carbon dioxide. This resultant lead carbonate, if not removed, will contaminate sections. The stain must therefore be filtered, immediately before use, either through a No 50 filter paper or through a syringe fitted with a Millipore Swinnex filter containing an MF millipore filter with 0.2 µm diameter pores. If a Swinnex filter is used the solution may conveniently be stored in the syringe and filtered solution expelled when required. After staining, the grids should be washed first in 0.02 M NaOH, which will help to remove any stain contamination and then in several changes of distilled water. These solutions should also be filtered as described above.

Masked metachromasia (Solcia et al, 1968)

Paraffin sections of 6% glutaraldehyde fixed tissues are brought to water and hydrolysed for 5 to 20 mins in 5 M hydrochloric acid at 60°C. After washing briefly in distilled water they are stained for 2 to 3 mins in 0.1% toluidine blue in acetate buffer pH 5.0. Dehydrate, clear and mount.

Lead haematoxylin (Solcia et al, 1969a)

Paraffin sections of 6% glutaraldehyde fixed tissues are brought to water and stained in Solcia's lead haematoxylin* for 2 to 3 hrs at 37°C. The depth of staining can be controlled visually by examining the sections under a microscope during the third hour. Rinse in distilled water. Dehydrate, clear and mount.

* Stabilised lead solution.

1) Add equal parts 5% aqueous lead nitrate and saturated aqueous ammonium acetate. Filter and add 2 ml of 40% formaldehyde to each 100 ml filtrate. Store (indefinitely) at 4°C.

2) Dissolve 0.2 g haemotoxylin in 1.5 ml 95% ethanol. Add 10 ml stock stabilised lead solution and dilute with 10 ml distilled water. Agitate for 30 mins then filter. Make up to 50 ml with distilled water.

Argentaffinity (Solcia et al, 1969b)

Paraffin sections of 6% glutaraldehyde fixed tissues are brought to water and stained for 30 to 45 mins at 60°C in preheated ammoniacal silver solution* Depth of staining can be controlled visually by examining the sections under a microscope. The slides are then washed in distilled water and fixed briefly in 0.5% sodium thiosulphate. Dehydrate, clear and mount.

* Ammoniacal silver nitrate.

Add 0.880 sp grav ammonia to 5% aqueous silver nitrate until the brown precipitate is just clear. Then add more 5% silver nitrate dropwise until the solution once again becomes slightly turbid.

Argyrophilia (Grimelius, 1968)

Paraffin sections of Bouin fixed tissues are brought to water and treated for 3 hrs at 60°C in aqueous silver nitrate (30 mg $AgNO_3$ in 100 ml acetate buffer pH 5.6). Then transfer sections directly to freshly prepared reducing solution* at 60°C. Control this reduction microscopically. Wash sections in distilled water, dehydrate, clear and mount.

* Reducing solution.

Add 1g hydroquinone ($C_6H_4(OH)_2$) and 5 g anhydrous sodium sulphite to 100 ml distilled water.

Aldehyde fuchsin (Bussolati and Bassa, 1974)

Paraffin sections of Bouin, or 6% glutaraldehyde fixed tissues are brought to water and treated for 2 hr at room temperature with a freshly made solution obtained as follows. Mix 40 ml 0.5 M ammonium hydroxide, 40 ml 0.025 M copper sulphate (adjusted to pH 9.0 by adding a few drops of 0.880 sp grav ammonia) and 10 ml of 0.2 M sodium metabisulphite. The sections are then washed in running tap water and rinsed in distilled water. The sections are then stained for a few mins in aldehyde fuchsin*, the depth of staining being controlled microscopically. After several rinses in 95% alcohol the sections should be dehydrated, cleared and mounted.

* Aldehyde fuchsin.

Add 1 ml hydrochloric acid and 1 ml paraldehyde to 100 ml of 0.5% basic fuchsin in 70% alcohol. Keep the solution overnight at room temperature until the mixture darkens to deep violet. The stain works more rapidly and strongly when fresh but will keep for some time.

<u>Toluidine blue stain</u> (Huber et al, 1968)

Sodium tetraborate (borax) 1 g

Toluidine blue 1 g

Distilled water 100 ml

Dissolve the borax (which raises the final pH to about pH 11, and assists dye penetration) in the distilled water and then add the toluidine blue, with constant stirring. When completely dissolved filter and store in an amber dropping bottle. Sections are stained by covering them with a few drops of staining solution and heating them for half a minute in a spirit burner flame. The stain should not be allowed to boil as this may cause the sections to lift. Excess stain is then washed off in running water, air dried and then either viewed direct or mounted in DPX.

TISSUE PROCESSING - EM

Fixation: all fixation should be performed using cold solutions (4°C).

1) Prepare marine fixative and cool to 4°C.

2) Transfer small pieces of tissue, not larger than 1 mm^3 to the marine fixative and leave for 2 hrs.

3) Transfer tissue to two changes of cacodylate buffer (pH 7.2) over 1 hr.

4) Transfer tissue to minimal quantity of osmium tetroxide 1% in cacodylate buffer (pH 7.2) for 1 hr.

5) Transfer to cacodylate buffer (pH 7.2) and leave overnight.

Dehydration: all dehydration should be performed using cold solutions (4°C).

Transfer the tissue through the following series of alcohols for the stated times:

30% alcohol	15 min
50% "	15 min
70% "	30 min
90% "	30 min
100% "	30 min
fresh 100% "	30 min
propylene oxide	10 min
fresh propylene oxide	10 min

EMBEDDING

Prepare TAAB Laboratories epoxy resin as per their instructions. Remove tissue from cold propylene oxide and transfer to the following mixtures, at room temperature (in a fume hood):

resin : propylene oxide	1 : 1	3 hrs
resin : propylene oxide	3 : 1	overnight
resin : propylene oxide	7 : 1	3 hrs
resin : propylene oxide	15 : 1	3 hrs
transfer the tissue to fresh resin		3 hrs
transfer the tissue to fresh resin		overnight

transfer the tissue to fresh resin in silicone rubber moulds and polymerise for 48 hrs at 60°C.

APPENDIX 2

To record the response to each dose, ten consecutive measurements of the volume of pancreatic secretion were recorded, at 5 min intervals. To determine the response the 'volume of total, or additional, response' was determined as follows. The average basal rate was determined as the average of the first three and the last measurement (-10, -5, 0 and 35 mins). This average value is then subtracted from each of the six outputs recorded over the 30 min period following dose administration.

Significance has been estimated by comparing the basal response (-10, -5, 0 and 35 mins) with the evoked response (5,10,15,20,25,30) by Student's t-test, using program t-distribution, statistics-10 from the Casio program library and a Casio FX-501P programming calculator.

RAT BIOASSAY

a) Boots secretin: 500 μg

Time	a (μl)	b (μl)	c (μl)	d (μl)	average (μl)
-10	6.25	6.75	5.5	6.25	6.2
-5	6.25	6.7	5.4	6.0	6.1
0	6.25	6.75	5.5	6.25	6.1
5	10.75	10.6	9.25	10.5	10.2
10	26.4	20.5	15.75	22.25	21.2
15	20.4	24.75	20.25	28.0	23.1
20	14.25	15.0	14.0	15.25	14.6
25	9.1	9.25	8.5	9.0	9.0
30	7.5	6.0	6.0	6.5	6.5
35	6.5	6.5	5.5	6.25	6.2
			additional o/p		47.8 μl

Boots secretin: 50 μg

Time	a (μl)	b (μl)	c (μl)	d (μl)	e (μl)	f (μl)	average (μl)
-10	7.0	8.75	4.25	4.5	7.5	4.4	6.6
-5	7.0	9.0	4.5	4.25	7.5	4.5	6.1
0	7.0	9.0	4.5	4.5	4.4	6.2	5.9
5	10.0	19.75	10.5	9.25	12.75	7.5	11.6
10	18.1	18.5	20.1	19.5	14.5	14.25	17.5
15	12.25	11.2	17.4	13.3	10.0	12.25	12.7
20	8.5	10.5	8.4	7.7	8.75	5.75	7.0
25	6.9	10.1	4.75	6.25	8.25	5.75	7.0
30	7.0	9.5	4.0	5.0	7.75	5.0	6.4
35	7.0	9.1	4.5	4.25	7.5	4.75	6.2
					additional o/p		27.1 μl

Boots secretin: 10 μg

Time	a (μl)	b (μl)	c (μl)	average (μl)
-10	5.1	5.0	8.5	6.2
-5	5.0	5.0	8.5	6.2
0	5.0	5.0	8.25	6.1
5	8.25	7.5	11.5	9.1
10	13.25	12.0	14.0	13.1
15	8.3	9.5	10.25	9.4
20	6.0	6.9	9.5	7.5
25	5.5	5.25	8.5	6.4
30	5.0	5.0	8.5	6.2
35	5.0	5.0	8.5	6.2
		additional o/p		17.7 μl

Boots secretin: 5 μg

Time	a (μl)	b (μl)	c (μl)	average (μl)
-10	5.0	6.5	7.25	6.3
-5	4.8	6.5	7.0	6.1
0	5.0	6.75	7.0	6.3
5	10.0	10.25	9.25	9.8
10	7.0	9.5	10.25	8.9
15	6.5	8.5	9.0	8.0
20	5.75	7.5	8.0	7.1
25	5.25	7.0	7.25	6.3
30	5.0	6.5	7.25	6.3
35	5.0	6.5	7.25	6.3
		additional o/p		9.3 μl

Boots secretin: 1 μg

Time	a (μl)	b (μl)	c (μl)	average (μl)
-10	5.5	4.5	6.75	5.6
-5	5.6	4.5	6.75	5.6
0	5.5	4.5	6.5	5.5
5	6.25	5.5	7.75	6.5
10	6.0	4.75	6.75	5.8
15	5.75	4.25	6.25	5.4
20	5.5	4.25	6.5	5.4
25	5.5	4.5	6.5	5.5
30	5.5	4.5	6.6	5.5
35	5.5	4.5	6.5	5.5
		additional o/p		0.63 μl

b) Porcine duodenal extract:

Dose Time (mins)	50 μg (μl)	100 μg (μl)	200 μg (μl)	400 μg (μl)	800 μg (μl)
-10	5.5	5.75	5.8	5.5	5.5
-5	5.5	5.5	5.5	5.5	5.6
0	5.5	5.5	5.5	5.5	5.5
5	8.5	11.5	16.75	17.75	25.0
10	8.0	11.75	11.25	16.25	15.75
15	5.5	6.1	8.0	7.3	7.1
20	5.75	5.5	5.8	5.8	6.1
25	5.5	5.6	5.5	6.0	5.75
30	5.5	5.5	5.5	5.8	5.5
35	5.5	5.5	5.5	5.5	5.5
Additional Output	6.25	15.0	19.8	26.9	32.3μl

c) Porcine gastric extract:

1.0 mg/kg

Time	a (μl)	b (μl)	c (μl)	d (μl)	average (μl)
-10	7.75	6.25	6.1	4.1	6.1
-5	8.0	6.25	6.25	4.0	6.1
0	8.0	6.0	6.1	4.25	6.1
5	8.0	6.1	6.25	4.5	6.2
10	7.9	6.25	6.1	4.25	6.1
15	8.0	6.1	6.25	4.25	6.2
20	8.0	6.1	6.25	4.25	6.2
25	7.9	6.1	6.25	4.25	6.1
30	8.0	6.25	6.25	4.1	6.2
35	7.9	6.1	6.25	4.25	6.1

no additional o/p

2.0 mg/kg

Time	a (μl)	b (μl)	average (μl)
-10	9.1	4.25	6.7
-5	9.0	4.25	6.6
0	9.0	4.5	6.8
5	9.0	4.5	6.8
10	9.0	4.25	6.6
15	9.1	4.25	6.7
20	9.0	4.25	6.6
25	9.1	4.25	6.7
30	9.1	4.1	6.6
35	9.1	4.25	6.7

no additional o/p

d) Styela gut extract:

1.0 mg/kg

Time	a (μl)	b (μl)	c (μl)	d (μl)	average (μl)
-10	8.5	6.0	7.5	4.75	6.7
-5	8.0	6.1	7.5	4.9	6.6
0	8.25	6.1	7.5	4.75	6.7
5	8.6	6.0	7.5	4.9	6.8
10	8.4	6.25	7.5	5.0	6.8
15	8.4	6.1	7.25	4.9	6.7
20	8.25	6.1	7.25	4.9	6.6
25	8.5	6.0	7.25	4.9	6.7
30	8.25	6.0	7.25	4.9	6.6
35	8.25	6.25	7.25	5.0	6.7
			additional o/p		0.23μl

2.0 mg/kg

Time	a (μl)	b (μl)	c (μl)	d (μl)	average (μl)
-10	5.6	4.5	9.1	5.9	6.3
-5	5.5	4.75	9.25	5.6	6.3
0	5.5	4.75	9.25	5.9	6.3
5	6.5	5.0	9.5	5.9	6.7
10	6.0	4.75	9.25	5.9	6.7
15	5.4	4.5	9.25	5.4	6.1
20	5.25	4.75	9.25	5.4	6.2
25	5.5	4.75	9.1	5.9	6.3
30	5.4	5.0	9.1	5.6	6.3
35	5.5	4.75	9.0	5.6	6.2
			additional o/p		0.4 μl

5.0 mg/kg

Time	a (μl)	b (μl)	c (μl)	average (μl)
-10	5.0	6.5	5.25	5.6
-5	5.25	6.5	5.0	5.6
0	5.0	6.5	5.0	5.6
5	5.75	7.1	5.75	6.2
10	5.75	9.0	6.0	6.9
15	5.5	7.8	5.75	6.4
20	5.5	7.0	5.25	5.9
25	5.25	6.6	5.25	5.7
30	5.25	6.5	5.1	5.6
35	5.0	6.6	5.1	5.6
		additional o/p		3.1 μl

e) Styela pharynx extract:

5.0 mg/kg

Time	a (μl)	b (μl)	c (μl)	d (μl)	average (μl)
-10	5.25	6.1	6.0	9.1	6.6
-5	5.25	6.0	6.1	9.1	6.6
0	5.0	6.0	6.0	9.0	6.5
5	5.25	6.1	5.75	9.1	6.6
10	5.25	6.0	6.1	9.25	6.7
15	5.25	5.75	6.0	9.0	6.5
20	5.0	6.0	6.0	9.0	6.5
25	5.25	6.0	6.1	8,75	6.5
30	5.0	6.1	6.1	9.0	6.6
35	5.0	6.0	6.1	9.0	6.6

no additional o/p

TURKEY BIOASSAY

a) Boots Secretin:

0.5 mg/kg

Time	a (μl)	b (μl)	c (μl)	average (μl)
-10	14.6	10.1	12.7	12.5
-5	13.8	9.7	11.4	11.6
0	13.8	9.9	11.6	11.8
5	81.3	72.0	51.2	68.2
10	31.7	28.7	23.5	28.0
15	20.3	16.2	16.5	17.7
20	17.1	12.4	12.7	14.1
25	13.8	10.7	11.4	12.0
30	8.9	9.9	12.7	10.5
35	14.6	9.9	12.1	12.2
		additional o/p		78.9 μl

1.0 mg/kg

Time	a (μl)	b (μl)	c (μl)	average (μl)
-10	17.1	10.5	12.1	13.2
-5	20.3	10.1	12.1	14.2
0	18.7	10.5	12.7	14.0
5	99.2	91.7	81.2	90.7
10	77.2	82.0	58.0	72.4
15	26.0	21.8	17.4	21.7
20	24.0	16.5	13.2	18.0
25	16.3	11.2	12.7	13.4
30	17.1	10.1	12.7	13.3
35	19.5	10.5	12.1	14.0
		additional o/p		160.5 μl

2.0 mg/kg

Time	a (µl)	b (µl)	c (µl)	average (µl)
-10	16.3	9.7	13.4	13.1
-5	14.6	9.7	12.7	12.3
0	15.4	9.2	13.8	12.8
5	143.1	126.2	92.3	120.5
10	108.1	88.0	101.4	99.2
15	43.1	38.4	35.0	38.9
20	32.5	31.1	28.2	30.6
25	22.8	22.8	18.1	21.2
30	27.6	14.2	13.8	18.5
35	17.9	9.2	13.4	13.5
		additional o/p		253.3 µl

b) Porcine duodenal extract:

2.0 mg/kg

Time	a (µl)	b (µl)	c (µl)	average (µl)
-10	10.4	12.2	10.1	10.9
-5	10.1	11.9	10.4	10.8
0	10.7	12.5	10.4	11.2
5	13.1	14.7	14.4	14.1
10	12.5	14.4	13.7	13.5
15	11.2	12.5	12.5	12.1
20	10.7	12.2	10.4	11.1
25	10.7	12.2	11.2	11.4
30	10.1	11.9	10.1	10.7
35	10.4	12.2	10.4	11.0
		additional o/p		7.1 µl

5.0 mg/kg

Time	a (µl)	b (µl)	c (µl)	average (µl)
-10	13.1	11.2	10.7	11.7
-5	12.8	10.o	9.9	10.9
0	12.5	10.5	9.9	11.0
5	24.4	28.7	19.5	24.2
10	16.3	19.5	21.8	19.2
15	14.4	14.6	16.3	15.1
20	12.8	10.7	11.2	11.6
25	12.5	11.2	12.7	12.1
30	12.5	12.2	10.5	11.7
35	12.8	10.5	10.1	11.7

additional o/p 26.7 µl

c) Porcine gastric extract:

5.0 mg/kg

Time	a (µl)	b (µl)	c (µl)	average (µl)
-10	14.7	10.7	11.2	12.2
-5	13.5	10.1	12.2	11.9
0	13.1	10.1	10.5	11.2
5	14.4	10.1	10.7	11.7
10	13.5	10.5	11.7	11.9
15	13.5	10.1	11.4	11.7
20	13.8	10.5	11.4	11.9
25	14.1	9.8	11.2	11.7
30	13.1	10.1	11.4	11.5
35	13.5	9.8	12.2	11.8

no additional o/p

d) Styela gut extract:

1.0 mg/kg | 2.0 mg/kg

Time	a (µl)	b (µl)	c (µl)	average (µl)	2.0 mg/kg a (µl)
-10	10.1	10.7	12.8	11.2	10.4
-5	10.1	10.7	13.1	11.3	7.9
0	10.1	11.2	13.1	11.5	9.9
5	14.3	9.8	12.8	12.3	9.3
10	7.2	9.8	12.5	9.8	9.9
15	10.7	10.7	13.1	11.5	9.6
20	9.5	9.8	13.4	10.9	9.9
25	10.7	9.4	13.1	11.1	10.4
30	10.7	9.8	13.4	11.3	9.9
35	10.7	9.8	13.1	11.2	9.9
		no additional o/p			no additional o/p

e) Styela pharynx extract:

2.0 mg/kg

Time	a (µl)	b (µl)	c (µl)	average (µl)
-10	11.8	12.7	13.5	12.7
-5	14.7	12.7	14.7	14.0
0	14.7	12.3	13.5	13.5
5	8.8	11.9	13.5	11.4
10	14.7	12.3	14.2	13.7
15	17.6	11.9	14.7	14.7
20	11.8	12.7	14.7	13.1
25	14.7	12.3	13.5	13.5
30	14.7	12.3	13.5	13.5
35	14.7	11.9	13.5	13.4
		no additional o/p		

APPENDIX 3

A standard curve was constructed by measuring a range of concentrations of bovine serum albumin (BSA) using the Lowry method. The absorbances were determined in the normal manner and a graph plotted of absorbance against protein concentration (Fig A1). This plot was linear over the range of values determined and, therefore, as all samples fell within this range, protein values could be extracted simply by multiplying the absorbance by a constant which was calculated to be 197.

ABSORBANCE (ABS)

BSA mg/dl	1	2	3	Mean	(mg/dl/ABS)
1	0.005	0.004	0.004	0.0043	233
2	0.007	0.011	0.010	0.0093	215
3	0.018	0.016	0.020	0.018	167
5	0.029	0.023	0.027	0.026	192
10	0.045	0.049	0.042	0.045	222
15	0.080	0.075	0.077	0.077	195
20	0.121	0.112	0.107	0.113	177
25	0.142	0.124	0.125	0.130	0192
30	0.164	0.152	0.156	0.160	187
40	0.196	0.188	0.201	0.195	205
50	0.267	0.256	0.274	0.266	187
				Average =	197

Fig A1

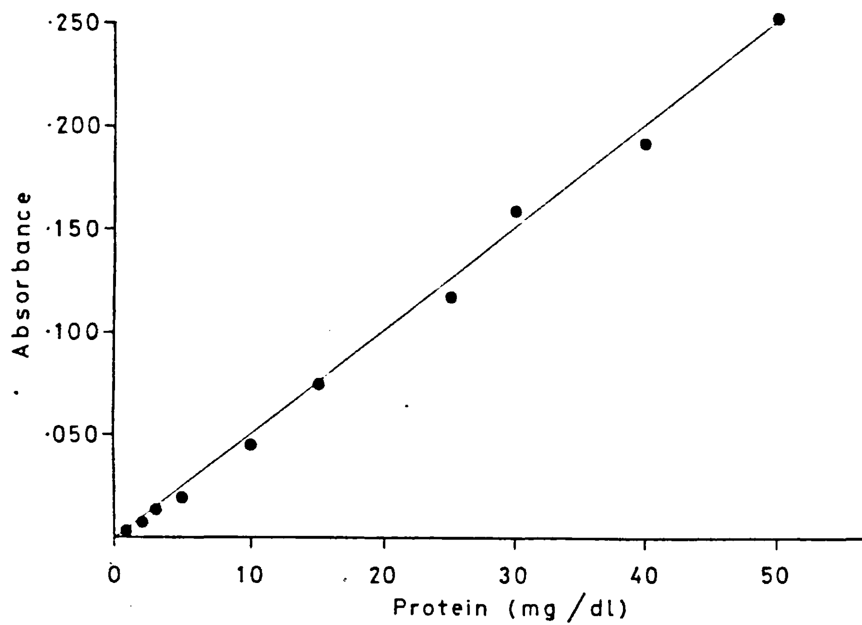


Fig A1 A standard curve of concentration of bovine serum albumin against absorbence, showing the range of concentrations over which linearity existed.

NO INFUSION

LOWRY PROTEIN, mg/dl:

	Ringer					Mean	± SD
-50	10.1	10.8	9.3	8.1	11.8	10.1	1.4
-40	7.5	9.2	7.1	5.5	10.1	7.9	1.8
-30	5.7	7.9	4.9	5.3	7.9	6.3	1.4
-20	4.3	5.5	5.5	2.6	3.0	4.2	1.4
-10	3.9	5.9	2.8	3.0	1.8	3.5	1.6
0	1.6	5.1	2.2	2.6	1.4	2.6	1.5
10	1.0	4.3	2.8	4.1	1.8	2.8	1.5
20	0.8	2.6	3.4	3.4	2.2	2.4	1.1
30	2.0	2.0	1.4	1.8	1.2	1.7	0.4
40	1.8	0.2	0.0	2.2	0.8	1.1	1.0
50	0.4	0.8	2.2	3.6	0.4	1.5	1.4
60	0.6	0.8	1.0	1.4	0.0	0.7	0.5

ACID PHOSPHATASE, U/1:

	Ringer					Mean	± SD
-50	2.2	1.8	1.3	2.7	1.9	1.98	0.52
-40	1.1	1.2	0.6	2.2	1.8	1.38	0.63
-30	0.8	0.7	0.3	1.5	1.1	0.88	0.45
-20	0.4	0.3	0.2	1.0	0.5	0.48	0.31
-10	0.4	0.2	0.3	0.8	0.4	0.42	0.23
0	0.1	0.4	0.3	0.4	0.3	0.30	0.12
10	0.4	0.2	0.2	0.3	0.1	0.24	0.11
20	0.4	0.3	0.1	0.4	0.4	0.32	0.13
30	0.4	0.0	0.2	0.1	0.3	0.13	0.12
40	0.1	0.1	0.0	0.0	0.o	0.06	0.05
50	0.3	0.1	0.1	0.2	0.0	0.14	0.11
60	0.4	0.0	0.1	0.1	0.3	0.18	0.16

CCK-8 (SULPHATED)

LOWRY PROTEIN, mg/dl

	10 μg			Mean	±SD	100 μg			Mean	±SD
-20	3.4	1.4	4.7	3.2	1.7	4.5	1.8	3.6	3.3	1.4
-10	2.6	1.6	2.8	2.3	1.6	2.6	4.6	3.0	3.4	1.0
0	1.6	1.4	2.4	1.8	0.5	2.4	4.0	2.4	2.9	0.9
10	4.9	12.8	4.3	7.4	4.7	17.2	25.8	13.6	18.9	6.3
20	2.6	4.9	1.2	2.9	1.9	6.1	13.2	7.5	8.9	3.8
30	2.0	2.6	0.6	1.7	1.0	5.9	3.6	4.7	4.7	1.2
40	1.2	2.2	1.4	1.6	0.5	2.6	1.8	3.0	2.4	0.6
50	1.6	1.2	0.8	1.2	0.4	2.6	0.6	1.6	1.9	1.1
60	0.2	1.6	1.4	1.1	0.7	1.8	1.0	2.2	1.6	0.6

LOWRY PROTEIN, mg/dl

	200 μg								Mean	±SD
-20	7.1	4.3	3.2	1.8	2.2	3.7	6.1	3.0	3.9	1.9
-10	9.7	5.5	1.4	1.6	2.8	2.4	2.0	1.4	3.3	2.9
0	5.1	4.5	2.0	1.8	1.4	0.6	1.0	1.0	2.2	1.7
10	20.5	17.8	25.8	12.0	10.9	31.0	38.7	34.9	23.9	10.4
20	16.2	5.3	19.7	11.2	25.6	20.9	6.9	14.2	15.0	7.0
30	12.4	3.6	2.4	2.4	5.5	9.7	4.9	5.5	5.8	3.6
40	13.0	3.0	0.2	2.2	4.3	5.5	3.4	5.3	4.6	3.8
50	4.9	2.0	0.0	1.0	4.3	4.0	1.8	3.4	2.7	1.7
60	4.1	3.9	1.4	0.8	2.6	3.9	1.6	3.0	2.7	1.3

CCK-8 (NON-SULPHATED)

LOWRY PROTEIN, mg/dl

	100 μg					Mean	±SD
-20	6.9	2.6	2.0	3.7	3.2	3.7	1.9
-10	5.3	2.2	1.2	3.0	3.2	3.0	1.5
0	3.7	1.0	2.2	1.6	2.4	2.2	1.0
10	13.8	18.9	14.8	9.3	24.5	19.8	8.7
20	12.0	11.0	5.7	16.4	9.9	11.0	3.8
30	5.5	4.3	3.7	4.5	8.1	5.2	1.7
40	6.3	1.4	2.4	2.6	2.4	3.0	1.9
50	3.7	2.6	2.4	1.0	1.8	2.3	1.0
60	3.9	2.6	1.8	2.8	1.6	2.5	0.9

ACID PHOSPHATASE, U/l

	100 μg					Mean	±SD
-20	0.5	0.7	0.2	0.9	0.3	0.5	0.3
-10	0.6	0.7	0.2	0.3	0.0	0.4	0.3
0	0.3	0.9	0.4	0.6	0.2	0.5	0.3
10	1.2	6.0	4.8	7.6	4.8	4.9	2.4
20	0.4	2.6	3.4	4.6	2.4	2.7	1.6
30	0.3	0.5	0.3	0.6	1.3	0.6	0.4
40	0.1	0.2	0.2	0.3	0.2	0.2	0.1
50	0.1	0.3	0.1	0.2	0.3	0.2	0.1
60	0.2	0.1	0.2	0.0	0.2	0.14	0.1

CCK-33

LOWRY PROTEIN, mg/dl

	10 μg				Mean	±SD
-20	6.1	5.1	2.8	4.9	4.7	1.4
-10	2.0	3.6	2.8	2.2	2.6	0.7
0	2.0	4.5	2.6	1.8	2.7	1.3
10	9.7	14.0	19.1	12.6	13.9	4.0
20	4.7	10.3	8.7	6.1	7.4	2.5
30	3.7	6.1	4.1	4.5	4.6	1.0
40	4.3	6.3	4.5	2.0	4.3	1.8
50	5.3	5.1	1.8	3.0	3.8	1.7
60	3.4	2.0	3.2	2.6	2.8	0.6

LOWRY PROTEIN, mg/dl

	100 μg					Mean	±SD
-20	1.0	5.3	3.4	6.9	3.2	3.9	2.3
-10	0.2	1.0	3.6	6.7	2.2	2.7	2.6
0	0.2	1.4	2.2	5.1	2.0	2.2	1.8
10	18.3	44.0	26.0	58.4	42.8	37.9	15.8
20	12.4	7.5	22.9	30.0	14.0	17.4	9.0
30	0.2	2.6	8.3	10.7	3.4	5.0	4.3
40	0	2.8	3.2	5.3	3.9	3.0	2.0
50	0	1.4	2.8	2.8	3.2	2.0	1.3
60	0.6	0.8	1.6	4.1	0.6	0.5	1.5

CCK-33 + 5 mM c-GMP

LOWRY PROTEIN, mg/dl

		10 μg		Mean	±SD
-20	4.0	4.3	5.1	4.5	0.6
-10	0.8	3.2	3.6	2.5	1.5
0	0.4	2.4	2.0	1.6	1.0
10	1.6	8.9	5.3	5.3	3.7
20	1.4	4.9	3.4	3.2	1.8
30	0.6	3.0	2.4	2.0	1.2
40	0.4	1.6	2.2	1.4	0.9
50	3.7	1.6	1.2	2.2	1.4
60	1.8	1.0	2.0	1.6	0.5

CCK-8 (SULPHATED) + 5 mM c-GMP

LOWRY PROTEIN, mg/dl

		10 μg			Mean	±SD
-20	5.7	4.1	3.6	4.9	4.5	1.0
-10	3.6	3.2	2.6	3.4	3.2	0.4
0	3.4	3.6	2.2	3.0	3.0	0.6
10	12.6	6.7	11.6	13.0	11.0	2.9
20	8.1	3.4	7.3	9.7	7.1	2.7
30	4.1	4.3	3.6	4.5	4.1	0.4
40	3.6	4.3	1.4	3.6	3.2	1.3
50	2.8	2.4	2.0	3.0	2.5	0.4
60	2.8	3.4	1.8	2.4	2.6	0.7

BOMBESIN

LOWRY PROTEIN, mg/dl

	0.1 μg		Mean	1.0 μg			Mean	±SD
-20	0.6	2.6	1.6	2.8	3.0	7.9	4.5	2.9
-10	0.4	2.0	1.2	2.0	3.4	5.5	3.6	1.8
0	1.0	1.2	1.1	1.8	2.0	3.6	2.4	1.0
10	8.5	3.7	6.1	15.0	19.5	10.3	14.9	4.6
20	1.8	1.4	1.6	4.5	6.5	7.1	6.1	1.3
30	1.4	1.0	1.2	1.8	3.6	3.4	2.9	1.0
40	0.8	0.8	0.8	2.4	3.2	3.2	2.9	0.5
50	0.8	0.6	0.7	1.6	1.6	3.0	2.0	0.8
60	0.4	0.0	0.2	2.2	1.2	2.0	1.8	0.5

LOWRY PROTEIN, mg/dl

	10 μg				Mean	±SD
-20	2.0	2.8	3.4	5.13	3.3	1.3
-10	2.8	1.2	2.4	2.6	2.2	0.7
0	2.2	0.8	1.8	1.8	1.6	0.6
10	17.2	26.2	23.9	18.2	24.4	8.7
20	9.7	9.5	16.2	8.3	10.9	3.6
30	4.9	5.1	5.5	4.5	5.0	0.4
40	2.8	3.6	3.0	1.6	2.7	0.8
50	1.2	2.2	2.0	2.6	2.0	0.6
60	1.2	2.0	2.8	1.6	1.9	0.7

PHYSALAEMIN

LOWRY PROTEIN, mg/dl

	100 μg				Mean	±SD
-20	6.5	2.4	5.7	3.2	4.4	2.0
-10	2.8	1.6	1.8	2.0	2.0	0.5
0	2.2	2.0	2.0	1.8	2.0	0.2
10	26.6	7.3	10.9	8.2	13.3	9.0
20	12.6	5.3	4.5	4.7	6.8	4.0
30	6.9	3.3	3.6	2.8	4.1	1.9
40	4.7	3.6	2.0	1.6	3.0	1.5
50	3.0	2.8	1.8	1.2	2.2	0.8
60	1.8	2.0	1.6	0.8	1.5	0.5

CAERULEIN

LOWRY PROTEIN, mg/dl

	100 μg			Mean	±SD
-20	3.6	3.2	5.7	4.1	1.4
-10	2.2	1.2	2.4	1.9	0.6
0	1.2	0	2.2	1.1	1.1
10	7.1	12.2	4.9	8.1	3.7
20	5.5	6.5	4.3	5.5	1.1
30	3.6	2.6	3.4	3.2	0.5
40	1.6	1.6	2.8	2.0	0.7
50	1.8	0.6	2.6	1.6	1.0
60	1.0	1.0	2.0	1.3	0.6

SECRETIN

LOWRY PROTEIN, mg/dl

	100 μg				Mean	±SD
-20	3.2	4.8	7.1	2.6	4.4	2.0
-10	2.4	2.6	5.7	1.8	3.1	1.8
0	2.6	2.2	4.6	4.0	3.3	1.1
10	3.6	2.2	0.4	1.6	1.9	1.3
20	3.4	2.0	1.2	2.0	2.1	0.9
30	2.0	1.2	4.6	2.6	2.6	1.4
40	2.0	0.8	5.0	1.4	2.3	1.8
50	2.4	0.4	2.8	0.6	1.5	1.2
60	1.6	0.4	0.8	0.0	0.7	0.7

ACID PHOSPHATASE, U/l

	100 μg				Mean	±SD
-20	0.5	0.6	1.2	0.4	0.68	0.36
-10	0.3	0.6	1.0	0.6	0.63	0.29
0	0.1	0.6	1.0	0.2	0.48	0.41
10	0.4	0.8	0.8	0.2	0.55	0.30
20	0.8	0.3	0.8	0.6	0.63	0.24
30	0.9	0.4	0.4	0.0	0.43	0.37
40	0.6	0.3	0.7	0.2	0.45	0.24
50	0.9	0.0	0.3	0.4	0.40	0.37
60	0.4	0.0	0.7	0.3	0.35	0.29

SECRETIN

LOWRY PROTEIN, mg/dl

	100 μg				Mean	±SD
-20	3.2	4.8	7.1	2.6	4.4	2.0
-10	2.4	2.6	5.7	1.8	3.1	1.8
0	2.6	2.2	4.6	4.0	3.3	1.1
10	3.6	2.2	0.4	1.6	1.9	1.3
20	3.4	2.0	1.2	2.0	2.1	0.9
30	2.0	1.2	4.6	2.6	2.6	1.4
40	2.0	0.8	5.0	1.4	2.3	1.8
50	2.4	0.4	2.8	0.6	1.5	1.2
60	1.6	0.4	0.8	0.0	0.7	0.7

ACID PHOSPHATASE, U/1

	100 μg				Mean	±SD
-20	0.5	0.6	1.2	0.4	0.68	0.36
-10	0.3	0.6	1.0	0.6	0.63	0.29
0	0.1	0.6	1.0	0.2	0.48	0.41
10	0.4	0.8	0.8	0.2	0.55	0.30
20	0.8	0.3	0.8	0.6	0.63	0.24
30	0.9	0.4	0.4	0.0	0.43	0.37
40	0.6	0.3	0.7	0.2	0.45	0.24
50	0.9	0.0	0.3	0.4	0.40	0.37
60	0.4	0.0	0.7	0.3	0.35	0.29

GLUCAGON

LOWRY PROTEIN, mg/dl

	100 µg				Mean	±SD
-20	3.7	5.3	3.2	2.0	3.6	1.4
-10	3.2	1.4	2.8	1.8	2.3	0.8
0	0.8	0.8	2.0	2.0	1.6	0.6
10	2.2	0.8	1.6	2.6	1.8	0.8
20	1.2	0.6	1.0	1.8	1.1	0.5
30	1.0	0.8	1.0	1.6	1.1	0.3
40	2.0	1.6	0.4	1.0	1.2	0.7
50	1.2	1.6	0.8	0.8	1.1	0.4
60	0.8	1.6	1.2	0.6	1.0	0.4

SIGMA CCK (TECHNICAL)

LOWRY PROTEIN, mg/dl

	0 μg					Mean	±SD
-50	7.5	10.0	11.3	8.1	12.5	9.9	2.1
-40	5.5	8.1	7.7	7.5	7.7	7.3	1.0
-30	4.9	6.5	5.1	4.1	4.7	5.1	0.9
-20	4.1	3.0	4.1	3.7	3.3	3.7	0.5
-10	2.6	2.2	3.9	3.3	2.2	2.8	0.8
0	1.0	2.0	3.0	3.5	1.4	2.2	1.1
10	1.4	1.2	2.8	3.2	0.6	1.8	1.1
20	0.6	1.8	2.2	2.6	1.0	1.6	0.8
30	1.0	1.0	2.0	2.6	0.4	1.6	0.8
40	1.2	0.8	0.8	3.5	0.0	1.3	1.3
50	0.6	0.0	1.8	0.4	0.2	0.6	0.6
60	0.6	0.0	3.3	0.8	0.2	1.0	1.4

ACID PHOSPHATASE, U/l

	0 μg					Mean	±SD
-50	1.6	1.4	1.9	1.5	2.0	1.68	0.26
-40	1.1	1.0	0.8	0.5	1.8	1.04	0.48
-30	0.8	0.4	0.7	0.2	1.1	0.64	0.35
-20	0.2	0.6	0.4	0.2	0.5	0.38	0.18
-10	0.3	0.4	0.5	0.0	0.5	0.34	0.21
0	0.2	0.3	0.2	0.4	0.3	0.28	0.08
10	0.5	0.2	0.2	0.0	0.3	0.24	0.18
20	0.4	0.1	0.0	0.1	0.4	0.20	0.19
30	0.4	0.0	0.2	0.2	0.3	0.22	0.15
40	0.1	0.1	0.3	0.0	0.0	0.10	0.12
50	0.3	0.1	0.0	0.1	0.2	0.14	0.11
60	0.4	0.0	0.1	0.1	0.3	0.18	0.16

SIGMA CCK (TECHNICAL)

LOWRY PROTEIN, mg/dl

	100 μg				Mean	±SD
-50	13.0	10.2	8.7	11.6	10.9	1.9
-40	8.2	8.1	4.7	7.5	7.1	1.6
-30	4.9	7.1	2.2	6.3	5.1	2.2
-20	4.5	3.9	2.4	5.1	4.0	1.2
-10	3.0	4.5	1.6	4.1	3.3	1.3
0	3.0	3.5	1.6	3.2	2.8	0.9
10	3.5	5.1	6.1	6.5	5.3	1.3
20	4.3	7.0	6.3	3.5	5.4	1.5
30	4.3	5.6	4.3	5.9	5.1	0.9
40	3.5	5.3	3.7	3.0	3.9	1.0
50	3.0	4.1	2.8	2.0	3.0	0.9
60	3.0	1.0	3.0	0.6	1.9	1.3

ACID PHOSPHATASE, U/l

	100 μg				Mean	±SD
-50	1.3	1.5	2.3	1.5	1.65	0.44
-40	0.6	0.5	1.7	0.7	0.88	0.56
-30	0.3	0.2	0.8	0.4	0.43	0.26
-20	0.2	0.0	0.8	0.2	0.30	0.35
-10	0.3	1.0	0.4	0.4	0.53	0.32
0	0.3	0.3	0.5	0.2	0.33	0.13
10	0.4	0.5	0.6	0.6	0.53	0.10
20	0.5	0.3	0.2	0.5	0.38	0.15
30	0.4	0.2	0.3	0.5	0.35	0.13
40	0.2	0.3	0.1	0.3	0.23	0.10
50	0.0	0.3	0.1	0.1	0.13	0.13
60	0.1	0.1	0.0	0.1	0.08	0.05

SIGMA CCK (TECHNICAL)

LOWRY PROTEIN, mg/dl

	200 μg					Mean	±SD
-50	10.2	10.8	13.4	8.1	7.5	10.0	2.4
-40	8.1	8.5	12.2	5.1	6.1	8.0	2.7
-30	6.5	7.1	8.3	5.5	5.1	6.5	1.3
-20	4.3	3.7	5.1	4.5	3.5	4.2	0.6
-10	1.8	3.3	3.9	3.5	4.1	3.3	0.9
0	1.0	3.5	2.0	3.0	3.2	2.5	1.0
10	15.2	10.0	20.1	17.5	10.2	14.6	4.4
20	11.8	8.5	15.0	16.0	9.1	12.1	3.4
30	8.1	5.3	7.5	12.6	7.1	8.1	2.7
40	5.1	4.1	6.5	7.3	4.1	5.4	1.4
50	4.3	3.5	5.5	6.9	3.5	4.8	1.4
60	3.7	3.5	4.3	4.1	1.8	3.5	1.0

ACID PHOSPHATASE, U/l

	200 μg					Mean	±SD
-50	2.3	1.7	1.9	1.8	1.3	1.8	0.36
-40	1.2	0.8	1.7	1.0	0.9	1.12	0.36
-30	0.8	0.7	0.8	0.5	0.6	0.68	0.13
-20	0.4	0.4	0.4	0.3	0.4	0.38	0.04
-10	0.4	0.5	0.2	0.2	0.3	0.32	0.13
0	0.1	0.4	0.2	0.2	0.3	0.24	0.11
10	0.9	0.6	0.5	0.8	0.7	0.7	0.16
20	0.7	0.6	0.2	0.5	0.4	0.48	0.19
30	0.5	0.2	0.2	0.5	0.4	0.36	0.15
40	0.4	0.1	0.3	0.1	0.2	0.22	0.13
50	0.0	0.3	0.1	0.3	0.2	0.18	0.13
60	0.2	0.1	0.0	0.1	0.0	0.08	0.08

SIGMA CCK (TECHNICAL)

LOWRY PROTEIN, mg/dl

	400 μg				Mean	±SD
-50	10.6	9.1	12.0	8.1	9.9	1.7
-40	8.5	7.4	5.9	8.9	7.7	1.3
-30	7.1	6.3	3.5	4.7	5.4	1.6
-20	4.1	3.2	2.6	5.5	3.8	1.3
-10	3.3	4.1	2.8	5.9	4.0	1.4
0	2.6	3.5	1.6	4.9	3.2	1.4
10	23.6	12.6	18.1	16.0	17.6	4.6
20	20.1	15.6	22.1	6.5	16.1	6.9
30	16.9	7.1	16.0	11.0	12.8	4.6
40	6.5	4.3	5.9	6.5	5.8	1.0
50	6.7	3.0	5.5	5.7	5.2	1.6
60	4.5	2.2	3.5	4.1	3.6	1.0

ACID PHOSPHATASE, U/l

	400 μg				Mean	±SD
-50	1.8	2.1	1.1	1.0	1.50	0.54
-40	1.2	0.8	0.6	0.4	0.75	0.34
-30	0.7	0.4	0.2	0.3	0.40	0.22
-20	0.3	0.4	0.1	0.1	0.23	0.15
-10	0.2	0.2	0.0	0.0	0.10	0.12
0	0.4	0.0	0.2	0.5	0.28	0.22
10	1.2	1.7	0.2	2.5	1.40	0.96
20	0.6	1.5	0.5	1.5	1.03	0.55
30	0.4	0.8	0.5	0.9	0.65	0.24
40	0.5	0.4	0.5	0.4	0.45	0.06
50	0.0	0.4	0.3	0.4	0.28	0.19
60	0.5	0.0	0.1	0.3	0.23	0.22

SIGMA CCK (TECHNICAL)

LOWRY PROTEIN, mg/dl

	100 μg				Mean	±SD
-50	12.0	16.4	10.2	9.9	12.1	3.0
-40	7.5	7.9	7.1	5.7	7.0	0.9
-30	6.5	7.1	5.3	6.3	5.8	1.2
-20	4.7	4.5	4.3	2.2	3.9	1.2
-10	4.1	3.0	3.3	1.8	3.1	1.0
0	2.6	3.0	3.2	1.0	2.4	1.0
10	37.2	34.9	26.2	38.6	34.2	5.6
20	48.5	42.8	49.3	28.6	42.3	9.6
30	34.1	25.0	32.1	21.1	28.1	6.1
40	18.1	13.2	15.6	10.6	14.4	3.2
50	21.0	12.0	8.5	4.9	9.4	3.4
60	9.1	10.8	7.1	2.2	7.3	3.7

ACID PHOSPHATASE, U/1

	1000 μg				Mean	±SD
-50	1.8	1.0	1.2	0.8	1.45	0.41
-40	0.9	0.6	0.6	0.9	0.75	0.17
-30	0.2	0.5	0.5	0.7	0.48	0.21
-20	0.0	0.4	0.3	0.5	0.30	0.22
-10	0.4	0.3	0.7	0.4	0.45	0.17
0	0.1	0.4	0.4	0.2	0.23	0.15
10	2.0	0.7	2.5	2.0	2.05	0.33
20	2.2	2.2	5.8	5.2	3.85	1.92
30	0.3	0.4	3.6	3.2	2.38	1.20
40	1.1	0.9	1.0	3.0	1.50	1.00
50	0.8	1.1	0.4	1.2	0.88	0.36
60	1.0	0.8	0.6	1.0	0.85	0.19

APPENDIX 4

Amino acid abbreviations:

Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
Cys	cysteine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
His	histidine
Ile	isoleucine
leu	leucine
Lys	lysine
Met	methionine
Phe	phenylalanine
Pro	proline
Ser	serine
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
Val	valine

APPENDIX 5

Abbreviations:

ACh	acetylcholine
c-AMP	3':5'-cyclic monophosphate
Bov	bovine
BSA	bovine serum albumin
Bt_2c-GMP	dibutyryl guanosine 3':5'-monophosphate
chick	chicken
CCK	cholecystokinin
CCK-8	cholecystokinin octapeptide
CSV	cardio-stomacic vessel
CTIP	concentrate of thermostable intestinal peptides
DOPA	dihydroxyphenylalanine
E-L	endocrine-like
FIF	formaldehyde-induced fluorescence
FITC	fluorescein isothiocyanate isomer 1
G-17	gastrin heptadecapeptide (1-17)
-GDP	-glycerphosphate dehydrogenase
GIP	gastric inhibitory peptide
GRP	gastrin releasing peptide
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
IP	intraperitoneal
PAP	peroxidase anti-peroxidase
PBS	phosphate buffered saline
PHI	peptide with n terminal histidine and c terminal isoleucine
PHM	peptide with n terminal histidine and c terminal tyrosine
Por	porcine
PYY	peptide with n terminal tyrosine and c terminal tyrosine
PI	phosphatidylinositol
RER	rough endoplasmic reticulum
SER	smooth endoplasmic reticulum
VIP	vasoactive intestinal peptide

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Endocrine Cells in the Oesophagus of the Ascidian *Styela clava*, a Cytochemical and Immunofluorescence Study

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Summary. Immunocytochemical studies have demonstrated the occurrence of an insulin-immunoreactive cell type in the oesophageal epithelium of the Ascidian *Styela clava*. Staining with aldehyde fuchsin has demonstrated a number of similar small, triangular, cells located on the basement membrane, which may have an endocrine function. Argyrophilic cells have also been found, suggesting the presence of a second endocrine cell type. The absence of argentaffin cells has led us to believe that the cells so far observed do not produce biogenic amines such as 5-HT (5-Hydroxytryptamine). The nature of these cells is discussed with reference to endocrine-like cells found in the digestive tracts of other protochordates.

Key words: Endocrine cells – Gut epithelium – Ascidian (*Styela clava*) – Insulin-like immunoreactivity – Light microscopy.

Introduction

Sufficient histological, cytochemical and ultrastructural evidence now exists to provide a good scheme for classification of endocrine cells in the gut of higher vertebrates. (Andrew 1976a, b; Capella et al., 1969; Pearse, 1969, 1971, 1974; Polak et al., 1976; Solcia et al., 1973, 1975.) These cells share a number of cytochemical and ultrastructural characteristics, possibly due to their common origin in neuroectodermal tissue (Pearse, 1969). The discovery of cells with similar characteristics in the digestive tracts of the lower vertebrates (Ostberg et al., 1976; Van Noorden and Pearse, 1976; Van Noorden, et al., 1972) and invertebrates (Fritsch, 1976; Fritsch and Sprang, 1977; Fritsch et al., 1976; Kataoka and Fujita,

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1974; Thorndyke and Bevis, 1977; Van Noorden and Pearse, 1976) has led to the suggestion that these cells may also produce polypeptide hormones.

However, convincing immunological evidence in favour of this contention is limited in invertebrates to the description of cells with glucagon-like, gastrin-like or insulin-like immunoreactivity in Amphioxus. (Van Noorden and Pearse, 1976) and insulin-like immunoreactivity in the gut of the bivalve *Mytilus edulis* (Fritsch et al., 1976).

The present study was undertaken subsequent to the observation of endocrine-like cells in the oesophagus of *Styela clava* (Thorndyke and Bevis, 1977), in an attempt to determine the nature of their products.

Materials and Methods

Specimens of *Styela clava* collected in the Portsmouth area were transported to London in aerated seawater, and transferred to circulating sea water aquaria at 10° C. The oesophagus was rapidly excised from each animal, transferred to Bouin fixative for 3 h and followed by dehydration in graded ethanols and standard paraffin wax embedding and sectioning. These sections were then stained for argyrophilia (Grimelius, 1968), argentaffinity (Solcia et al., 1969), masked metachromasia (Solcia et al., 1968), aldehyde fuchsin or immunofluorescence.

Immunofluorescence. Paraffin sections (6 µ) were transferred to albuminised slides, dried overnight at 37° C, and then immersed in xylene and petroleum ether to remove the wax. Immunofluorescence staining was by the indirect fluorescent antibody technique (Coons et al., 1955). The sections were treated with anti serum to bovine insulin (Wellcome Reagents Ltd., England) produced in guinea pig, and diluted 1 : 10 with 0.01 M phosphate buffered saline (PBS) pH 7.0. Following incubation for 48 h at 4° C, the slides were washed in PBS, treated for 1 h at room temperature using FITC conjugated swine anti-guinea pig serum (Nordic Immunological Laboratories) and diluted 1: :10 with PBS. The slides were again washed in several changes of PBS, mounted in buffered glycerine (9 parts glycerine to 1 part PBS) and examined with a Zeiss Universal Epi-fluorescent photomicroscope. Control staining was carried out using a non-immune serum.

Results

Immunofluorescence. Positive immunofluorescent staining was observed throughout the oesophagus, these cells appearing to extend upwards from a broad base on the basement membrane to a slender process reaching towards the gut lumen (Fig. 1 a).

Cytochemistry. Aldehyde fuchsin-positive cells were found in the same position as immunofluorescent cells, but they were seen to be far more numerous (Fig. 1 b). The dense population of oesophageal mucous cells was also stained, but as these invariably occupy an apical position adjacent to the gut lumen, they do not interfere with observation of cells on the basal lamina.

Tests for argentaffinity gave negative results as did methods for masked metachromasia. However, Grimelius' method for argyrophilia proved positive (Fig. 1 c), staining distinctly granular cells which also rested on the basement membrane.

Fig. 1a–c. Light photomicrographs showing oesophageal epithelium of *Styela clava*. **a** Insulin-immunoreactive cell (arrow) following immunofluorescence staining with antiserum to insulin. × 1160. **b** Aldehyde fuchsin stained cell (arrow) resting on the basement membrane. × 650. **c** Argyrophilic cell (arrow), showing distinct granules clustered towards the base of the epithelium, the intervening unstained area being due to the plane of section. × 1250. *BM* basement membrane, *L* lumen, *M* apical mucous cells

Discussion

This present demonstration of insulin-like immunoreactivity in the oesophagus of the Ascidian *Styela clava* supports the initial findings of insulin immunoreactivity in gut extracts of *Ciona* (Davidson et al., 1971; Falkmer, 1969 Falkmer and Wilson, 1967), and the more recent insulin immunofluorescence observed in *Amphioxus* (Van Noorden and Pearse, 1976). In this latter case, as in the present study, cross reactivity was weak, and positive staining only obtained using more extreme conditions than those normally employed with mammalian tissues.

This however, might be anticipated since immunofluorescence is a highly specific technique, and protochordate polypeptides could be expected to be immunologically quite different from their mammalian counterparts. Nevertheless, there must be some similarities for even weak staining to have taken place. It is possible that anti-chicken insulin, if available, might produce better results in tunicates, as Ascidian insulin, extracted from the gut of *Ciona intestinalis* has been found to be antigenically more similar to chicken insulin than mammalian insulins (Falkmer and Wilson,1967).

The numbers, appearance and position on the basement membrane of the aldehyde fuchsin-positive cells described in the present study match the distribution and appearance of the granular oesophageal cells of *Styela* seen at the ultrastructural level (Thorndyke and Bevis, 1978). In *Amphioxus*, insulin immunofluorescent cells have been shown to be also aldehyde fuchsin-positive (Van Noorden and Pearse, 1976). However, the immunofluorescent cells in *Styela* oesophagus are observed less frequently than aldehyde fuchsin-positive cells, this may be due to difficulties in observing small, weakly fluorescing cells, or it may represent a real differentiation amongst aldehyde fuchsin-positive cells, only some of them producing an immunoreactive insulin-like factor. In this regard it is of interest that only a single granular cell type (Type II) can be distinguished in *Styela* oesophagus at ultrastructural level (Thorndyke and Bevis, 1978), thus any difference is necessarily a subtle one.

In *Amphioxus*, glucagon/gastrin immunoreactive cells have been shown to be argyrophilic (Van Noorden and Pearse, 1976), thus the finding of argyrophilic cells in *Styela* oesophagus may indicate that *Styela* type II cells include glucagon gastrin-like cells as well as insulin-like cells, the difference being indistinguishable at the ultrastructural level. A similar situation obtains in *Ciona* where both argentaffin and argyrophilic cells have been reported (Fritsch, 1976), while ultrastructural evidence points to only a single endocrine-like cell type (Fritsch and Sprang, 1977). It seems that only further work with anti-glucagon and anti-gastrin will show whether the argyrophilic cells indeed represent a genuine third endocrine cell type in *Styela*, as suggested for *Amphioxus* (Van Noorden and Pearse, 1976).

Despite general agreement that the endocrine-like cells found in protochordates may produce a polypeptide hormone or hormones (Biuw and Hulting, 1971; Burighel and Milanesi, 1975; Kataoka and Fugita, 1974; Fritsch and Spräng, 1977; Thorndyke and Bevis, 1978; Van Noorden and Pearse, 1976), it is as yet unknown whether they play a real physiological role in these animals. It may be that these cells, and their hormone-like products are simply homologues of the vertebrate endocrine system with no major physiological function existing for them

at this primitive evolutionary stage. It is perhaps relevant that the gastropod mollusc, *Strophocheilus oblongus*, a primitive protostome, has been shown to metabolise glucose loads more rapidly following treatment with insulin (Marques and Falkmer, 1976). Clearly, a similar series of experiments in protochordates would prove an invaluable aid in the interpretation of current cytochemical reports. Preliminary investigations in *Ciona intestinalis* (Bevis and Thorndyke, unpublished observations) show that a glucose induced hyperglycaemia can be produced, and thus an investigation of this nature should be feasible.

In conclusion, the present study supports the initial indications of an insulin producing endocrine cell, located in *Styela* oesophagus (Thorndyke and Bevis, 1978). It further suggests the possibility of a second, argyrophilic, oesophageal endocrine cell, which as yet has not been distinguished at ultrastructural level. These results further support the need for intensified research in this area of key evolutionary importance, in order to promote a clear picture of the development of the complex, vertebrate gastrointestinal endocrine system.

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GENERAL AND COMPARATIVE ENDOCRINOLOGY 55, 251–259 (1984)

Comparative Studies on the Effects of Cholecystokinins, Caerulein, Bombesin 6-14 Nonapeptide, and Physalaemin on Gastric Secretion in the Ascidian *Styela clava*[1]

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The effects of cholecystokinins (CCK), caerulein, bombesin 6-14 nonapeptide (bombesin), and physalaemin on gastric secretion in *Styela clava* were measured using a perfusion technique. Varying concentrations of both CCK_{33} and CCK_8 produced a significant dose-dependent response. Dose for dose, CCK_{33} was more potent than CCK_8, while the assay was unable to show any discrimination between sulphated and nonsulphated forms of CCK_8. The specific CCK inhibitor Bt_2cGMP significantly reduced the response to both CCK_{33} and CCK_8. Both caerulein and physalaemin were effective although with a considerably reduced response compared with CCK. Bombesin was the most potent of all secretagogues tested while glucagon was without effect on gastric secretion. It is suggested that the primitive prepancreatic zymogen cells in *Styela* possess a receptor or receptors with an ability to recognise those peptides which are also active on vertebrate pancreatic acinar cells. It is further suggested that while the results indicate a receptor system less sophisticated than that found in vertebrates, they also imply the presence of an endogenous polypeptide hormone or hormones with a sequence which might be expected to show similarities to more than one vertebrate gastrointestinal peptide.

In recent years the role of secretagogues in the control of acinar cell zymogen secretion in the mammalian exocrine pancreas has been the subject of intense investigation (Gardner and Jensen, 1980; Gardner *et al.*, 1980; Jensen and Gardner, 1979; Jensen *et al.*, 1978, 1981; Schulze and Stolze, 1980). Such studies have shown that the mammalian pancreatic acinar cell carries a number of specific receptors which fall naturally into at least six classes according to their ability to interact with various secretagogues and specific inhibitors. In this way receptor classes have been identified which bind peptides from the secretin, cholecystokinin (CCK), bombesin 6-14 nonapeptide (bombesin), and substance P families as well as those responsive to acetylcholine and cholera toxin (Gardner and Jensen, 1980; Jensen and Gardner, 1979; Jensen *et al.*, 1978, 1981). Detailed investigation of the structural requirements for the action of CCK and related peptides on enzyme secretion in the mammalian pancreas highlight the importance of the sulphated tyrosine at position 27 (position 7 from the C terminus). Thus, desulphation or a change in position of the sulphated tyrosine from 7 to 6 (as in gastrin) considerably reduces potency although not always efficacy (Gardner *et al.*, 1980). Such subtle alterations in sequence allow for the sophisticated target specificity achieved in mammals where peptides of the CCK/gastrin complex are also involved in the control of gall bladder contraction and gastric acid secretion (Dockray, 1979, 1981).

In lower vertebrates the role of peptides

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in the regulation of gastrointestinal function is less clearly defined. In particular, the interrelationships between peptide hormones of the classes noted above and the evolution of endocrine control of pancreatic enzyme secretion have received relatively little attention. Studies on the most ancient vertebrates, the agnatha, indicate a role for CCK in the stimulation of gut enzyme secretion which predates the evolutionary appearance of the exocrine pancreas as a discrete and separate organ (Vigna and Gorbman, 1979). This secretory response was noted in the absence of any effect on gut or gall bladder smooth muscle activity. Further support for a primitive role of CCK in the regulation of prepancreatic gut secretion came in a recent study on the stimulation by CCK of gastric enzyme secretion in the protochordate ascidian *Styela clava* by the present authors (Bevis and Thorndyke, 1981). Such studies strongly support the idea of the early emergence of an endocrine (or perhaps paracrine) control of digestive enzyme secretion in chordates. However, more complete information concerning the responses of protochordate prepancreatic zymogen cells to the established secretagogues of mammalian acinar cells is lacking. This present study, therefore, investigates in some detail the response of such prepancreatic zymogen cells to a number of these secretagogues.

MATERIALS AND METHODS

Materials. (*1*) Animals. Specimens of *S. clava* were collected in the Portsmouth area and transported to London in aerated seawater. Animals were subsequently maintained in a fasting condition in a circulating seawater aquarium at 10°.

(*2*) *Hormones.* Bombesin 6-14 nonapeptide, caerulein (caeruletide), and physalaemin were the kind of gifts of Roberto de Castiglione, Farmitalia. Pure porcine CCK_{33} was a gift from Professor Viktor Mutt, Stockholm, and synthetic sulphated CCK_8 (Sincalide) was a gift from Squibb. Synthetic nonsulphated CCK_8 was purchased from Peninsula Laboratories Inc. and glucagon from Calbiochem. Dibutyryl cyclic GMP (Bt_2cGMP) was purchased from Sigma Chemical Co.

Experimental. The stomach of each specimen was perfused with artificial seawater (Synthetica) according to a procedure described previously (Bevis and Thorndyke, 1981). Artificial seawater was introduced through an oesophageal cannula at a flow rate of 28 μl/min and the perfusate collected as 10-min aliquots through a tube inserted through the intestine into the distal end of the stomach. Perfusate samples were collected over 2 h with the test infusion being given after 60 min. Freshly prepared test substances were infused in 200 μl of tunicate Ringer through a cannula placed in the cardio–stomaic vessel. A single dose of test peptide was given to each animal. Our previous studies (Bevis and Thorndyke, 1981) had shown that perfusate protein levels provided a convenient index of enzyme output and this method was, therefore, adopted in the present investigation. Furthermore, histological studies of postexperimental animals showed no evidence of mucous secretion or sloughing of cells, although they did indicate an increase in zymogen secretion. Thus perfusate protein was estimated by the Lowry method (Lowry *et al.*, 1951) in duplicate 100-μl fractions of each 10-min aliquot.

Full details of the experimental procedure and its rationale may be found in the earlier publication (Bevis and Thorndyke, 1981).

Statistical analysis. Differences between means were considered to be significant if, using Student's t test, $P < 0.05$. Values are expressed as the mean ± SEM.

RESULTS

All forms of CCK tested induced a significant elevation of perfusate protein (= enzyme) levels, with maximal levels occurring within 10–15 min of hormone administration (Figs. 1, 2). Recovery to near-basal levels took place within approximately 50 min, although at higher doses the elevated levels were more long lasting. It is notable that dose for dose, CCK_{33} was more potent than CCK_8. Maximum protein output with CCK_{33} at 2.6×10^{-5} and 2.6×10^{-6} M was 37.9 ± 7.9 and 13.9 ± 2.0 mg/dl, respectively, while somewhat higher doses of CCK_8, 1.1×10^{-4} and 1.1×10^{-5} M, increased protein levels to 18.9 ± 4.5 and 7.4 ± 3.3 mg/dl, respectively. Figure 3 shows, comparatively, the effect of similar (1.1×10^{-4} M) infusions of sulphated and nonsulphated CCK_8. The indications are that, in our hands, they are equipotent. In those experiments where the specific CCK

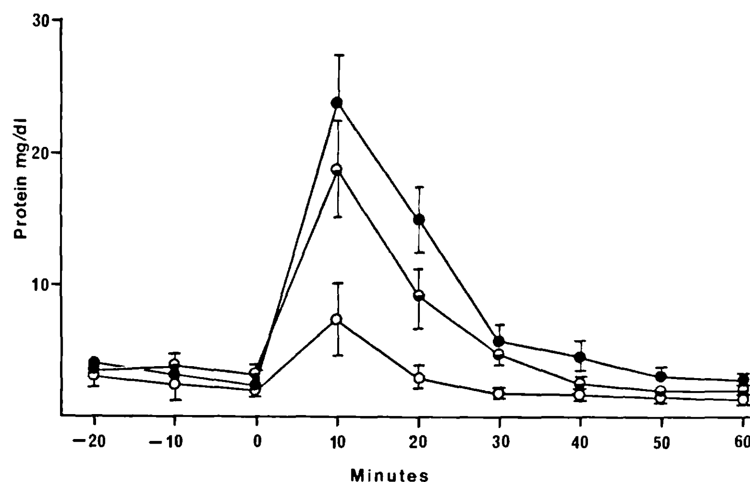

FIG. 1. Effect of sulphated CCK_8 on perfusate protein levels. Infusions of $1.1 \times 10^{-5} M$ (○, n = 3), $1.1 \times 10^{-4} M$ (◒, n = 3), and $22 \times 10^{-4} M$ (●, n = 8) CCK_8 all produced levels of perfusate protein significantly higher than controls. Mean ± SEM.

inhibitor Bt_2cGMP (5 m*M*) was inflused together with CCK_8 and CCK_{33} the elevation of perfusate protein levels was significantly reduced when compared to that achieved with either administered alone (Fig. 4). It is also worth noting that these data again confirm the greater effectiveness of CCK_{33} over CCK_8 (Sincalide). In this regard it would be of interest to investigate the effectiveness of CCK_4, the C-terminal fragment of which contains the active site for CCK and gastrin. Unfortunately CCK_4 was

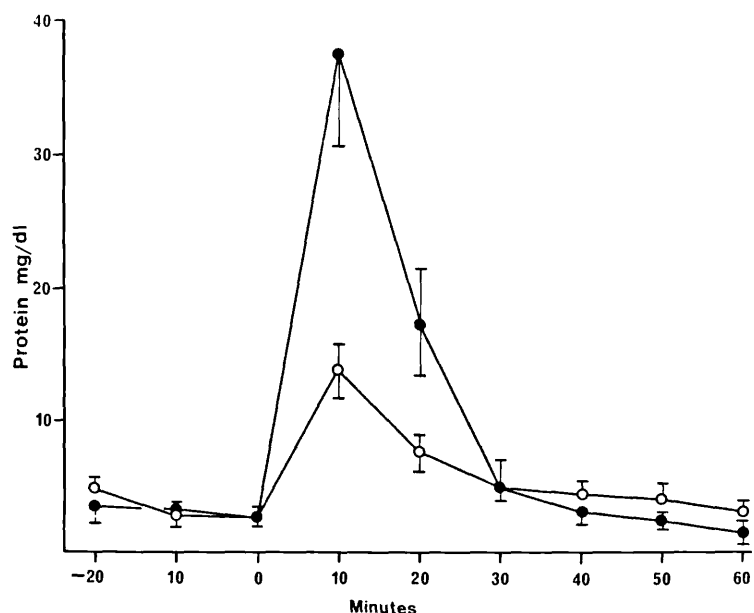

FIG. 2. Effect of CCK_{33} on perfusate protein levels. Infusions of $2.6 \times 10^{-6} M$ (○, n = 4) and $2.6 \times 10^{-5} M$ (●, n = 5) both produced levels of perfusate protein significantly higher than controls. Mean ± SEM.

not available for trial. The effect of Bt_2cGMP on bombesin- and physalaemin-induced secretion was not tested. Tests with physalaemin and caerulein were also effective but here the secretory response induced was lower than that seen with similar doses of CCK, although the time course of the response remained the same (Fig. 5). Thus, 7.7×10^{-5} *M* physalaemin and 7.3×10^{-5} *M* caerulein produced maximal levels of 13.3 ± 5.2 and 8.1 ± 2.6 mg/dl, respectively, within 10 min of administration.

The most effective of all secretagogues tested was bombesin. Here, treatment with 9×10^{-6} *M* bombesin induced a maximal level of 24.4 ± 5.0 mg/dl secretory protein within the first 10-min postinfusion period (Fig. 6). Trials with glucagon (2.9×10^{-5} *M*) produced no significant effect on gastric protein secretion (Fig. 5).

DISCUSSION

The results presented above indicate clearly that a number of vertebrate gastroenteropancreatic (GEP) peptides stimulate the release of gastric enzyme secretion in *S. clava,* measured as Lowry protein. This seems to imply the presence of a population of prepancreatic zymogen cells with a receptor complement able to recognise certain vertebrate gut peptide hormones (Figs. 1–6). In mammals, pancreatic acinar cells possess a sophisticated array of receptor classes, where acetylcholine, physalaemin (with substance P, eledoisin, and kassinin), bombesin (with litorin, ranatensin, and alytesin), secretin (with vasoactive intestinal peptide, VIP), and CCK (with caerulein and gastrin) each demand their own receptor class. There is, in addition, a sixth class with cholera toxin as the involved agonist.

Detailed investigation of structure–activity relationships within the CCK receptor class by Gardner and his colleagues (Gardner and Jensen, 1980; Gardner *et al.*, 1980; Jensen *et al.*, 1981) emphasise the importance of the sulphated tyrosine residue at position 7 from the C terminus as a requirement for full potency. Desulphation or translocation from position 7 to 6 from the C terminus, as in gastrin, considerably reduces activity in the acinar cell system. In a similar way these alterations also curtail the gall bladder smooth-muscle-contracting or cholecystokinetic effect of CCK, while at the same time conferring greater efficacy in the stimulation of gastric acid secretion as the molecule becomes more gastrin-like.

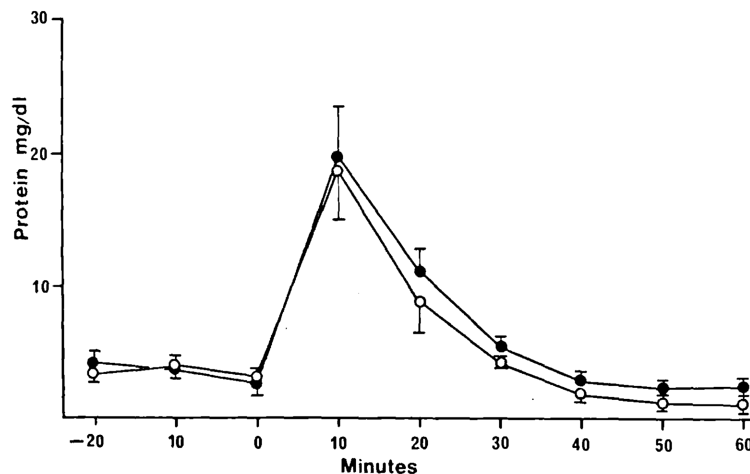

FIG. 3. Comparative effects of 1.1×10^{-4} *M* each, sulphated (○, $n = 3$) and nonsulphated (●, $n = 3$) CCK_8. No significant difference in response was observed. Mean ± SEM.

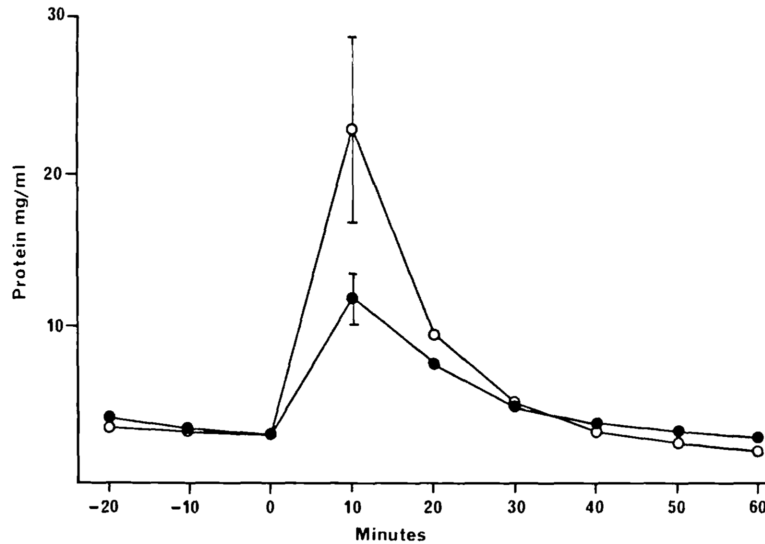

FIG. 4. Effect of $1.1 \times 10^{-4} M$ sulphated CCK_8, with (●, $n = 4$) and without (○, $n = 4$) Bt_2cGMP (5 m*M*). This specific CCK inhibitor significantly reduces the normal CCK response. Mean ± SEM.

Another unique feature of the CCK receptor class is the efficiency of the competitive antagonist Bt_2cGMP. Presence of this moiety specifically inhibits the interaction of CCK and structurally related forms with its receptor (Dockray, 1981; Dockray *et al.*, 1980; Jensen *et al.*, 1981; Peiken *et al.*, 1979). In lower vertebrates the situation is less well known, largely due to the lack of published investigations in this field. In the agnatha, as in ascidians, there is no discrete exocrine pancreas and zymogen cells may be found scattered sporadically in the intestinal mucosa (Barrington, 1972). Vigna and Gorbman (1979) showed that, in *Eptatretus*, the secretion of lipase from the intestinal cells could be induced by porcine CCK_{33} while secretin, VIP, and glucagon were without effect. Agonists for other mammalian acinar cell receptor classes were not tested. It is an attractive hypothesis that this could represent a primitive action of CCK before the evolutionary development of the pancreas as a separate organ and indeed it seems likely that this gastrointestinal role for CCK predates its cholecystokinetic effect since, when tested on smooth muscle prepara-

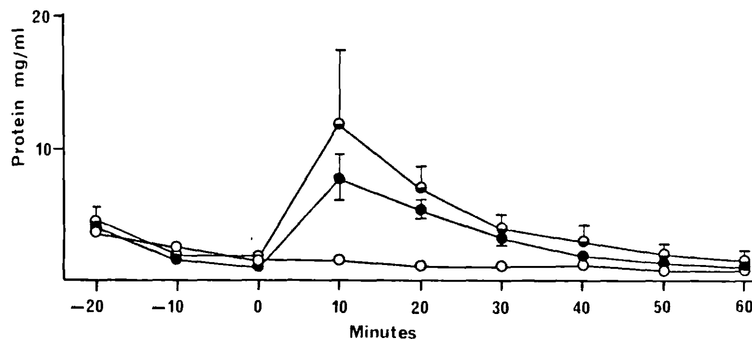

FIG. 5. Comparative effects of physalaemin, $7.7 \times 10^{-5} M$ (◒, $n = 4$), caerulein, $7.3 \times 10^{-5} M$ (●, $n = 3$), and glucagon, $2.9 \times 10^{-5} M$ (○, $n = 4$). Both physalaemin and caerulein induced a significant increase in perfusate protein levels, while glucagon was without effect. Mean ± SEM.

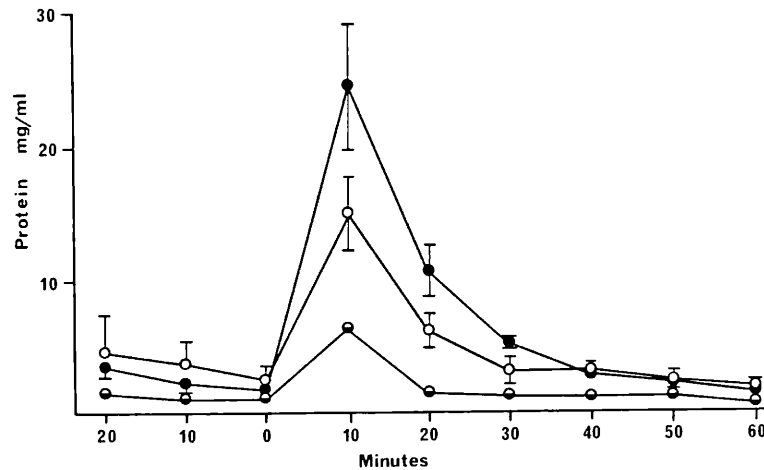

FIG. 6. Effect of bombesin (bombesin 6-14 nonapeptide) on perfusate protein levels. Infusions of 9 × $10^{-6}M$ (●, n = 4), 4.5 × $10^{-6}M$ (○, n = 3), and 4.5 × $10^{-7}M$ (◒, n = 3) all increased perfusate protein levels. Mean ± SEM.

tions from *Eptatretus* gall bladder and intestine, CCK failed to induce any contractions although acetylcholine was fully effective (Vigna and Gorbman, 1979). Support for such a primitive role for CCK comes from previous studies on ascidians by the present authors. In these animals there is neither discrete pancreas nor gall bladder homologue or indeed any gut-associated smooth muscle (Bevis and Thorndyke, 1981). In this way control of gastrointestinal cell secretion may well be the major regulatory role for such GEP peptides as are present. It should be noted here that the terms "stomach" and "gastric," as applied to ascidians, are used largely as a matter of convenience and should not be compared directly to their vertebrate counterparts. Thus, the stomach in *Styela* is a complex organ where both intra- and extracellular digestion take place. The gastric epithelium is characterized by mucus-producing cells, endocrine cells, absorptive cells, and a discrete population of prepancreatic zymogen cells (Thorndyke, 1977; Thorndyke and Bevis, 1978; Bevis and Thorndyke, 1979, 1981).

In our earlier study (Bevis and Thorndyke, 1981) we showed that porcine CCK and CCK_8 stimulated the release of secretory proteins in the *Styela* perfused stomach preparation (presumably from the prepancreatic gastric zymogen cells). The current investigation extends this work and suggests that even at this relatively early stage in chordate evolution, zymogen cells already possess the beginnings of the rich receptor complement seen in higher vertebrates and mammals in particular. Thus, these primitive zymogen cells appear to have receptors sensitive not only to a variety of CCK molecules and related peptides such as caerulein, but also to bombesin and physalaemin, each a representative agonist of a major receptor class in mammalian acinar cells (Figs. 5, 6). Moreover, this sensitivity has certain elements of the specificity seen in higher forms as evidenced by the ability of Bt_2cGMP to antagonise the CCK effect (Fig. 4). However, since studies on competitive effects between these peptide classes have not been undertaken, it is impossible to say, at this stage, to what extent we are dealing with a number of different receptor types or whether these animals have a single broadly specific receptor which identifies a range of GEP peptides. It is recognised, of

course, that the doses of peptides used in the present study do represent levels normally considered pharmacological in vertebrates, and in this respect further experiments on the effect of Bt_2cGMP on bombesin- and physalaemin-induced secretion could be instructive. However, it should be noted that there is a certain degree of specificity here since both glucagon (Fig. 5) and secretin (Bevis and Thorndyke, 1981) are totally without effect. Furthermore, the need to apply such doses should not be totally unexpected in an animal so far removed from the mammalian line. Clearly there is a lower level of zymogen cell sophistication in ascidians since they are apparently unable to distinguish sulphated and nonsulphated forms of CCK. This is in accord with a pattern of evolutionary progression whereby in fishes we do indeed see the beginnings of structure–activity relationships. Thus in coho salmon, studies on gall bladder activity show that although change in position of the sulphated tyrosine from 7 to 6 from the C terminus has little or no effect on activity, desulphation of this residue dramatically reduces CCK potency (Vigna and Gorbman, 1977). Such evolutionary changes are likely to be the result of a complex set of adaptive mechanisms, including the development of jaws together with a separate stomach. It is also likely that is was during this period in the evolutionary development of gastrointestinal regulatory mechanisms that a role for CCK/gastrin in the control of gastric acid secretion emerged (see Vigna, 1983). It was notable in our earlier study that secretin was without detectable effect on the primitive zymogen cells in *Styela* (Bevis and Thorndyke, 1981), although it remains a speculative possibility that secretin is concerned with the separate control of mucous cell secretion). This would imply that, notwithstanding our earlier comments suggesting the presence of a rather broadly specific receptor or receptor class on these prepancreatic zymogen cells, there is clearly an element of specialization in that, whatever the receptor complement may be on these cells, receptors akin to the secretin/VIP class or classes are evidently absent.

In mammals, acinar cell receptor classes may be grouped into super classes according to the second messenger employed. The secretin/VIP class is unique among the peptides in operating through a cAMP-dependent system; all remaining peptides utilize calcium as a second messenger. It is an attractive possibility to suggest that in evolutionary terms, receptors on primitive zymogen cells of the type found in ascidians invoke calcium as their intracellular messenger and that only later does cAMP become involved as part of the secretin/VIP receptor complex. Such a hypothesis is in accord with the present results and those from our earlier study, although it should be pointed out that we have not investigated the second messenger system in *Styela* zymogen cells and therefore this possibility remains speculative.

Finally, it is worth noting that receptor specificity and development go hand in hand with peptide hormone evolution and clearly the apparent lack of receptor sophistication in *Styela* zymogen cells could, in part, reflect the nature of the endogenous peptides or peptides for which the receptor or receptors have become adapted. In other words, this present report highlights the problem of hormone versus hormone receptor evolution. In our earlier publication (Bevis and Thorndyke, 1981) we noted that there was some difficulty in identification of the native ascidian peptide in that immunofluorescence studies indicated a secretinlike peptide rather than a CCK-like molecule. In spite of this difficulty it was suggested that the endogenous ascidian molecule might be an extended peptide with several sites of biological activity. Thus full receptor occupancy might require a number of distinct amino acid groupings. This point receives some support from the apparently greater effectiveness of CCK_{33}

over CCK_8 (Fig. 1, 2), and argues in *Styela* for receptors with a requirement for more than the C-terminal octapeptide sequence of CCK. Furthermore, recent collaborative studies using semipurified *Styela* gut extracts (Thorndyke, Bevis, Dimaline, Dockray and Mutt, unpublished observations) have shown that *Styela* gastric endocrine cells do indeed elaborate a peptide with rather more CCK-like than caerulein-like properties based upon bioassay and radioimmunoassay rather than immunocytochemistry alone. Indeed, indications are for a peptide somewhat larger than CCK_8. Moreover, when such partially purified extracts were tested in the *Styela* gastric assay preparation, they were found to be considerably more potent than any presently tested peptide (Thorndyke and Bevis, unpublished data). It could therefore be that the native peptide (or peptides), when fully characterised, will prove to have a composition and sequence which crosses the peptide class barrier and comes close to being the prosecgastrin molecule which has been the highly prized ancestral peptide sought by comparative gastrointestinal endocrinologists for a number of years. Indeed, mammalian bioassay of the same semipurified extracts referred to above do have secretin/VIP-like activity in addition to their CCK-like features (Thorndyke and Bevis, 1983). Studies currently underway in this and collaborating laboratories are going some way to answer these questions.

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A Cytochemical and Immunofluorescence Study of Endocrine Cells in the Gut of the Ascidian *Styela clava*

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Summary. Strong secretin-like immunofluorescence has been demonstrated in endocrine-like cells from the gastric epithelium of *Styela*. These cells also stain with lead haematoxylin and exhibit a brilliant formaldehyde-induced fluorescence, but do not show any other cytochemical features characteristic of the mammalian APUD series. Tests with antisera to glucagon, gastrin and somatostatin all proved negative. In the oesophagus tests with all four antisera proved negative. The significance of these results is discussed in relation to the phylogeny of vertebrate gastro-intestinal hormones.

Key words: Endocrine cells – Gut epithelium – Ascidian (*Styela clava*) – Immunofluorescence – Cytochemistry.

The general ultrastructural appearance and cytochemistry of endocrine-like cells in the gastrointestinal tract of protochordates is now becoming well documented (Bevis and Thorndyke, 1978; Burighel and Milanesi, 1975; Fritsch, 1976; Fritsch and Sprang, 1977; Fritsch et al., 1978; Thorndyke and Bevis, 1978; Van Noorden and Pearse, 1976). These basal granulated cells appear to elaborate peptides which have immunologically similar counterparts in the vertebrate gastro-entero-pancreatic endocrine system. Thus, cells showing insulin-like, glucagon-like, gastrin-like and somatostatin-like immunoreactivity have been demonstrated in the gut of several protochordate species (Bevis and Thorndyke, 1978; Fritsch et al., 1978; Van Noorden and Pearse, 1976).

No clear pattern has emerged and this present study is an attempt to clarify the situation, following the successful demonstration of insulin-like immunoreactivity

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in endocrine-like cells of *Styela* oesophagus (Bevis and Thorndyke, 1978) by testing for secretin, gastrin, glucagon and somatostatin immunoreactivity in one species, *Styela clava*.

Materials and Methods

Specimens of *Styela clava*, collected in the Portsmouth area, were transported to London in aerated seawater and transferred to circulating seawater aquaria at 10° C. Animals were rapidly dissected, small pieces of stomach and oesophagus excised and transferred either to Bouin fixative in seawater for 3 h or 6% glutaraldehyde. Following standard paraffin wax embedding, sections were cut, and stained for argyrophilia (Grimelius, 1968), argentaffinity (Solcia et al., 1969a), masked metachromasia (Solcia et al., 1968), lead haematoxylin (Solcia et al., 1969b), aldehyde fuchsin, or were used in immunofluorescence tests. Additional samples of stomach and oesophagus were freeze dried and formaldehyde vapour fixed (Falck et al., 1962) for the demonstration of biogenic amines by formaldehyde induced fluorescence (FIF).

Immunofluorescence. Antibodies to porcine secretin were raised in New Zealand white rabbits. The antigen was conjugated to bovine serum albumin by coupling with 1-ethyl-3 (3-dimethyl-aminopropyl) carbodiimide hydrochloride (Sigma) (Goodfriend et al., 1964). The product was then emulsified in Freund's complete adjuvant and injected in multiple subcutaneous sites at 0, 1 and 6 months. Bouin-fixed sections (6 µm) were transferred to albuminised slides, dried overnight at 37° C and then dewaxed in xylene and petroleum ether. Immunofluorescence staining was carried out according to the indirect antibody technique (Coons et al., 1955). Sections were treated with anti-porcine secretin serum prepared by the authors, or with anti-glucagon anti-synthetic human gastrin, anti-synthetic somatostatin or anti-porcine secretin; all produced in rabbits, and all the kind gift of Julia Polak and Susan Van Noorden, Royal Postgraduate Medical School, London. Dilutions with phosphate buffered saline (PBS) were in the range 1:50 to 1:10,000.

Control staining included the use of non-immune rabbit serum, PBS, or diluted immune serum preabsorbed overnight at 4° C with the appropriate antigen. Positive controls were also carried out using known positive mouse tissues.

Following incubation for 18 h in humid containers at room temperature, sections were washed in PBS and treated for 1 h with FITC conjugated swine anti-rabbit serum (Nordic) diluted 1:30 with PBS. Following a further wash in PBS the sections were mounted in buffered glycerine (9 parts glycerine to 1 part PBS) and examined with a Zeiss Universal Epi-fluorescence photomicroscope.

Results

Cytochemistry. Endocrine-like cells in the cap region of the gastric epithelium showed bright fluorescence with FIF (Fig. 1b) and positive staining with lead haematoxylin (Fig. 1c). Tests for argyrophilia, argentaffinity, masked metachromasia and aldehyde fuchsin were all negative. This is in direct contrast to the reactions of oesophageal endocrine-like cells whose cytochemistry is reported elsewhere (Bevis and Thorndyke, 1978).

Immunofluorescence. Gastric endocrine-like cells exhibited positive immunofluorescence with both anti-secretin preparations used (Fig. 1a). Brilliant, long, slender cells extending from basement membrane to gut lumen were observed at antiserum dilutions from 1:50 to 1:10,000. Positive control cells in mouse duodenum also showed strong fluorescence with these antisera but only at dilutions down to 1:1,000. No positive immunostaining was observed with anti-glucagon.

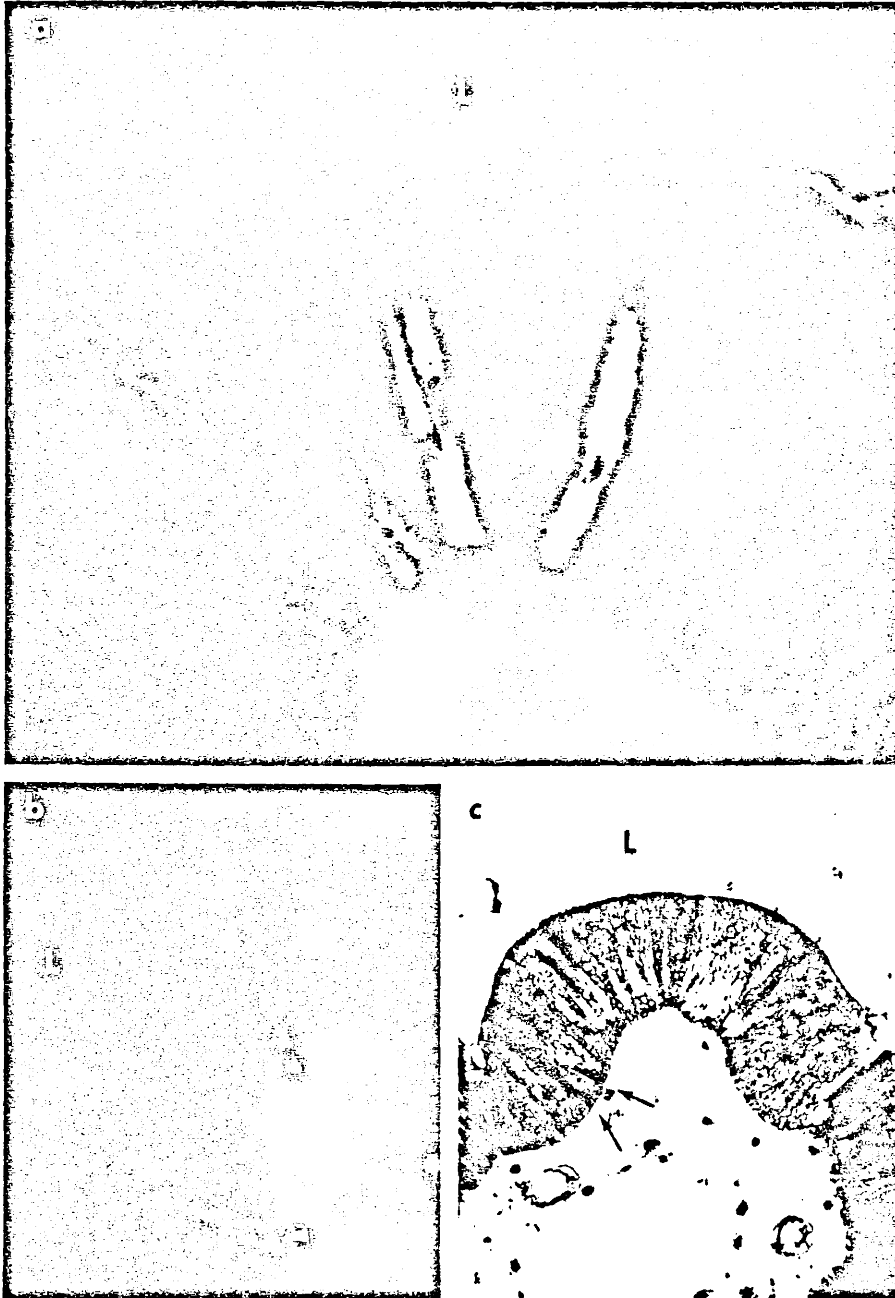

Fig. 1 a–c. Photomicrographs of the cap region from the gastric epithelium of *Styela clava*. **a** Immunofluorescence of cells after treatment with dilute anti-secretin serum. × 300. **b** Formaldehyde induced fluorescence. × 200. **c** Lead haematoxylin, × 200. *L*, gut lumen

anti-gastrin or anti-somatostatin although positive controls using mouse tissue in parallel proved positive.

In the oesophagus, no positive immunostaining was observed with any of the anti-sera tested.

Discussion

Earlier work has shown that the gastrointestinal tract of the ascidian *Styela clava* contains at least two types of granulated endocrine-like cell (Thorndyke and Bevis, 1978). Type II cells, which are restricted to the oesophageal epithelium, have been shown to exhibit insulin-like immunoreactivity together with a number of other characteristics typical of vertebrate gut endocrine cells (Bevis and Thorndyke, 1978).

This present study confirms the individuality of Type I gastric endocrine-like cells, which exhibit a brilliant secretin-like immunoreactivity together with various APUD characteristics quite distinct from those found in Type II cells. Furthermore, it is clear that the secretin-like immunoreactivity is specific since two quite distinct anti-secretin preparations were successfully used, while parallel studies using anti-glucagon, anti-gastrin and anti-somatostatin sera all proved negative. Similar studies using these four anti-sera on the oesophageal Type II cells of *Styela* were all negative.

This is in sharp contrast to the situation which obtains in *Ciona intestinalis*, where both gastrin-like and somatostatin-like immunoreactivity has been localised, while insulin-like and secretin-like factors are apparently absent (Fritsch et al., 1978). In addition, the gut of another, more advanced and perhaps more specialised protochordate, amphioxus, has been shown to include endocrine cells with insulin-like, glucagon-like and gastrin-like immunoreactivity (Van Noorden and Pearse, 1976).

Thus the gastrointestinal endocrine relations within this phylogenetically important group of animals seems far from clear and show a greater degree of divergence than might have been anticipated. This serves to highlight our inadequate knowledge of such invertebrate groups. Preliminary results from related ascidian studies (Thorndyke and Bevis, to be published) indicate that while secretin-like activity is found in *Dendrodoa grossularia*, it is absent from *Ascidiella aspersa*. Indeed, no other immunocytological demonstration of this hormone in lower chordates or invertebrates has been recorded since its demonstration in canine and human intestines (Polak et al., 1971 a–b).

Secretin-like activity has, however, been demonstrated by bioassay in intestinal extracts from lampreys (Barrington and Dockray, 1970; Nilsson, 1973). Unfortunately subsequent immunocytochemical tests failed to identify the site of secretin production (Van Noorden and Pearse, 1974).

Clearly our knowledge of vertebrate gastrointestinal hormone phylogeny is incomplete and schemes that have been presented include a degree of speculation. However, both morphological and biochemical evidence suggests that insulin, glucagon, gastrin and secretin may all have a common evolutionary origin (Adelson, 1971; Track, 1973). With the present demonstration of secretin-like

activity in *Styela*, all of these key gut hormones have now been located in the protochordate gastrointestinal tract. It must be noted, however, that some of these hormones are also found in protostomian phyla. Thus gastrin has been demonstrated immunologically in molluscs (Straus et al., 1975) and the presence of molluscan insulin has been demonstrated by various means (Davidson et al., 1971; Fritsch et al., 1976; Marques and Falkmer, 1976). Basal granulated endocrine-like cells have been observed in both bivalve and gastropod molluscs (Boquist et al., 1971; Fritsch and Sprang, 1977) and similar cells have been observed in the gastrointestinal system of *Macropipus puber* (Decapoda) (Thorndyke and Trinder, unpublished observations). These findings give support to the idea that the vertebrate gastrointestinal endocrine system may have originated in the primitive invertebrate gut, perhaps even before the separation of protostomian and deuterostomian lines.

In this regard it is of great interest that in recent studies on the differentiation of mammalian gut endocrine cells (Larsson and Jorgensen, 1978) it was found that secretin producing cells pass through a developmental stage where they also store a biogenic amine, as demonstrated by formaldehyde-induced fluorescence. At this stage in their differentiation then, these mammalian cells display a remarkable similarity to the secretin immunoreactive cells of *Styela*.

The gastric Type I cells of the present study show an unusually high immunological sensitivity, being ten times more sensitive to porcine secretin antibodies than were mouse control tissues. This may indicate an unusually high concentration of a secretin-like factor in *Styela*; equally it may represent a much closer antigenic similarity between *Styela* "secretin" and porcine secretin than might have been anticipated. Investigations currently in progress are designed to determine whether this secretin-like factor is also active in a bioassay.

As with the demonstration of insulin-like material in *Styela* (Bevis and Thorndyke, 1978) it is not known what role, if any, the secretin-like material might play in the animal's normal physiology. It would seem likely, however, that the production of such apparently large quantities of this material, in an active organ like the stomach, has some functional significance. Only a detailed analysis of ascidian physiology will answer these questions. Such a study is currently in progress in this laboratory.

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GENERAL AND COMPARATIVE ENDOCRINOLOGY 45, 458–464 (1981)

Stimulation of Gastric Enzyme Secretion by Porcine Cholecystokinin in the Ascidian *Styela clava*[1]

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Accepted February 3, 1981

The effects of porcine cholecystokinin (CCK) and secretin on gastric enzyme secretion in *Styela clava* were measured using a perfusion technique. Secretin was found not to be active, whereas CCK and CCK-octapeptide were both found to stimulate gastric release of acid phosphatase and proteins. Varying doses of CCK, between 0.1 and 1.0 mg, produced an approximately linear dose/response. It is suggested that these results are evidence of a paracrine system involving a prosecgastrin-like molecule, produced in the gastric epithelium.

Protochordates are the most primitive chordate group and occupy a unique position in vertebrate phylogeny. They possess some highly specialized adaptations while retaining a number of primitive features which makes their study important when considering the evolutionary development of vertebrate gastrointestinal peptide hormones. Two protochordate groups have so far been examined for peptide hormones using histochemical and cytochemical techniques, the cephalochordates (Branchiostomatidae) (Van Noorden and Pearse, 1976), and the more primitive urochordates (Ascidiacea) (Bevis and Thorndyke, 1978; 1979; Thorndyke and Bevis, 1978; Fritsch and Sprang, 1977; Fritsch *et al.*, 1978). Both groups possess cells which are immunologically similar to vertebrate peptide hormone-producing cells (Pearse, 1969). The cells, which extend to the gut lumen contain basal, electron-dense granules.

Using immunofluorescence techniques, glucagon-, gastrin-, and insulin-like peptides have been observed in the gut of amphioxus, *Branchiostoma lanceolatum* (Van Noorden and Pearse, 1976), and insulin-, gastrin-, secretin-, and somatostatin-like immunoreactivity has been observed in the guts of the ascidians *Styela clava* and *Ciona intestinalis* (Bevis and Thorndyke, 1978; 1979; Fritsch and Sprang, 1977; Fritsch *et al.*, 1978).

These findings indicate that the gut epithelia of these protochordate groups contain cells which produce peptides immunologically similar to some vertebrate gut hormones. However, there are not sufficient grounds at the present time for referring to a protochordate gut endocrine system, since there is no evidence that these peptides are biologically active in these animals.

If a protochordate gut endocrine system were to exist, then it would require methods other than immunofluorescence to demonstrate it. The present study approaches this question using a gastric perfusion technique.

MATERIALS AND METHODS

Materials

1) Animals. Specimens of *Styela clava* were collected in the Portsmouth area and transported to London in aerated seawater. Animals were subsequently maintained in a fasting condition in a circulating seawater aquarium at 10°. Most animals adapted well to these conditions, and the small percentage of moribund animals were readily identified by their

[1] A preliminary report on part of this work was given at the Third International Symposium on Gut Hormones in Cambridge, U.K., September 14–18, 1980 (Thorndyke and Bevis, 1980).

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flacidity, and closed siphons. Only healthy, turgid, actively siphoning animals were used in experiments.

2) Hormones. Synthetic, nonsulphated cholecystokinin-octapeptide and porcine secretin were purchased from Peninsula Laboratories, Inc., California. Technical grade CCK was purchased from Sigma (London).

Perfusion Technique

1) Anatomy. Styela clava is a sessile filter feeder and has a simple gut conveniently divided into four distinct regions: the pharynx, oesophagus, stomach, and intestine. However, it should be pointed out that these terms are used largely as a matter of convenience and should not be compared directly with their vertebrate counterparts. The short oesophagus draws food particles from the pharynx into the stomach, where both intracellular and extracellular digestion take place. The gastric epithelium is folded into a series of ridges, the apices of which form distinct "caps," which consist of mucous-producing cells, and endocrine-like cells (Fig. 1). The side walls contain absorptive cells, and enzyme-secreting zymogen cells. Underlying these ridges is a complex network of blood spaces, which connect to vessels lying on the outer surface of the stomach and leading to the general circulatory system. These gastric vessels all radiate from the cardiostomaic vessel (CSV) which connects directly to the simple heart.

Preliminary experiments using a resin-corrosion technique have shown that material infused via the CSV reaches the gastric blood spaces. As this is also the largest vessel in the animal, except for the very delicate heart, the CSV was used as the infusion site.

2) Surgical procedure (Fig. 2). A left ventral incision was made, extending the length of the body cavity, passing through all the layers of the body wall, and exposing the gut, without damaging the pharynx. A polyethylene tube (Portex 100) was inserted into the proximal end of the stomach through a small incision in the distal end of the oesophagus and secured with a suture. A large tube (Portex 300) was then inserted through a shallow incision on the outer side of the intestinal loop, passing into the distal end of the stomach, and secured by a ligature at the proximal end of the intestine. It was found that this tube could become occluded by the gastric epithelium, and to prevent this from occurring, a hole was cut in the side wall about 5 mm from the proximal end.

The animal was then transferred to a small Perspex dish through which was passed aerated seawater at 12°. Using the cannulae, the stomach was flushed with 5 ml of artificial seawater to remove any solid gastric contents, and then using a binocular microscope the CSV was located and loosely ligated. A small incision was made, and a short polyethylene tube (Portex 100), which had been drawn slightly to reduce the outside diameter at one end to 0.2–0.3 mm, was filled with tunicate Ringer and inserted with the aid of a micro-

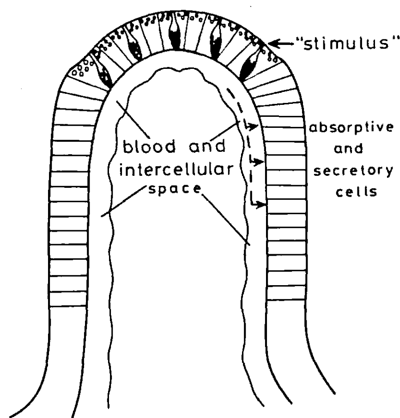

FIG. 1. Schematic representation of a transverse section of one of the gastric ridges. The cap regions project into the gastric lumen and consist of mucous cells as well as endocrine cells which are well situated to respond to material passing through the stomach. It is suggested that such a stimulus may cause the release of a hormonal factor into the underlying blood spaces. This could then stimulate basal receptors on the "zymogen" cells, causing them to release digestive enzymes into the gastric lumen.

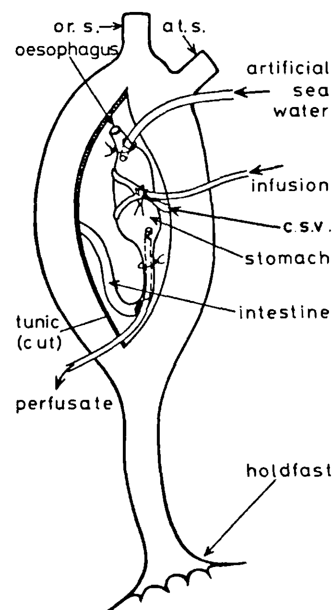

FIG. 2. Schematic representation of cannulae placement for gastric perfusion in *Styela*. Cannulation of esophagus and intestine provides gastric input and drainage, respectively, while cannulation of CSV affords a site for reproducible infusion of test material. at.s., Atrial siphon; or.s., oral siphon; CSV, cardiostomaic vessel.

manipulator. The ligature was then firmly tightened around the cannula.

With practice this procedure took about 10 min, occasional failures being caused by rupture of the vein during cannula insertion, or puncture of the gastric epithelium by the cannula tip.

Experimental Procedure

The stomach was perfused with artificial seawater (Synthetica), through the oesophageal cannula, using a Watson–Marlow pump (MHRK-1), at a flow rate of 28 μl/min. The perfusate was collected in the wells of a microtitre plate, the cannula being transferred to a fresh well at 10-min intervals.

After six samples had been collected, 200 μl of the tunicate Ringer, containing the test material, was infused through the CSV cannula over a 15-sec period. A short infusion period was chosen to make certain that adequate pressure was developed to ensure that hormones would come in contact with the basal edge of the gut epithelial cells.

Control animals were either cannulated but given no infusion, or infused with 200 μl of tunicate Ringer.

Samples were taken over a 2-hr period, the infusion being given after 1 hr. At the end of this period protein and acid phosphatase levels in each sample were determined.

Protein and acid phosphatase determination. Perfusate protein levels were taken as an index of enzyme output, and were measured using 0.2 ml of the seawater perfusate, by the Lowry method (Lowry *et al.*, 1951). However, it was found that addition of the Folin–Ciocalteu reagent (Sigma, London) caused a salt precipitate to form, which interfered with the absorbance measurements. Spinning the tubes in a bench centrifuge for the 30-min development period, however, caused the precipitate to be compacted, and a reliable absorbance value of the suupernatant could then be determined. This method gave a very reproducible standard curve, with values slightly lower than those obtained from protein solutions in water.

Acid phosphatase levels were determined using a colorimetric method (Boehringer Mannheim GmbH, W. Germany). All absorbances were measured on a Pye Unicam SP8-100 spectrophotometer.

Administration of hormones. The weights of individual animals are fairly variable when repeatedly weighed, as a significant quantity of seawater is retained in their pharynxes. A high percentage of the weight of these animals comprises seawater and the keratinous tunic, which is often covered with common fouling organisms. The wet weight of these animals was therefore felt to be an unreliable index for calculating the dose for each animal. Animals were therefore chosen which were of approximately equal size. The dry weights of these animals (less tunic) were found to lie within the range 0.51–1.22 g.

Hormones (0.1–1.0 mg) were dissolved immediately before use in filtered tunicate Ringer (NaCl, 25.9 g/liter; KCl, 0.75 g/liter; $CaCl_2$, 1.05 g/liter; $MgCl_2$, 5.295 g/liter; Na_2SO_4, 1.63 g/liter; $NaHCO_3$, 0.06 g/liter; and glucose, 1.0 g/liter).

Statistical analysis. Differences between means were considered to be significant if, using Student's *t* test, $P < 0.05$.

RESULTS

Effect of Seawater Perfusion on Protein and Acid Phosphatase Levels

Figure 3 shows the effect of perfusion with artificial seawater on the levels of protein and acid phosphatase in the perfusate. During the first 60-min, protein levels fall from 10 ± 1.5 mg/dl to 3.7 ± 1.8 mg/dl. In the absence of an infusion, protein output remains below this level throughout the following 60 min.

Acid phosphatase levels show a similar response, output in the perfusate falling from 2.0 ± 0.5 to 0.3 ± 0.1 units/liter after 60 min. Output then remains below this level over the next 60 min.

Infusion of tunicate Ringer without CCK (Fig. 4) caused no increase in either protein or acid phosphatase levels during the subsequent 60 min.

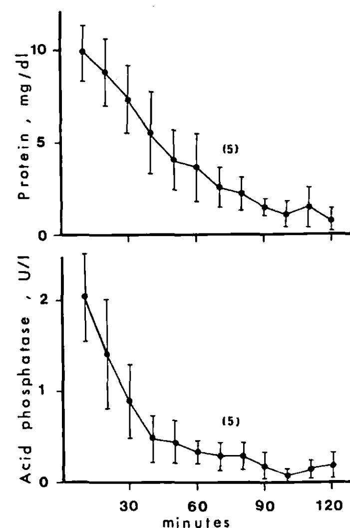

FIG. 3. Effect of seawater perfusion on protein and acid phosphatase levels in the perfusate over a (20-min period). Mean ± SE, $n = 5$. Stomachs are preflushed with 5 ml of seawater, and then perfused at 28 μl/min. Both protein and acid phosphatase levels show a decrease in perfusate levels over the following 120 min.

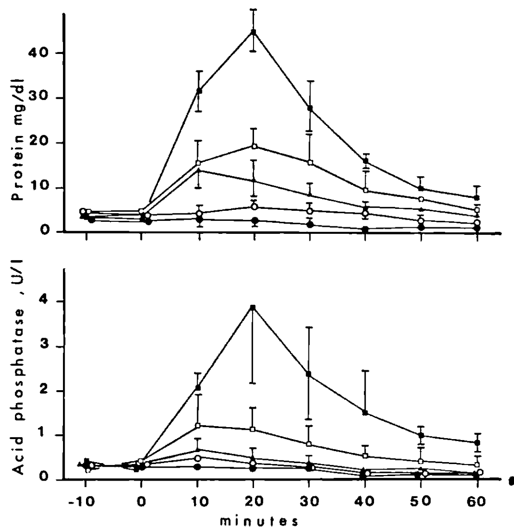

FIG. 4. Effect of Sigma CCK (infused at 0 min) on protein and acid phosphatase levels in the perfusate over the subsequent 60 min. Mean ± SE. Infusions of 0.1 mg (○, n = 4), 0.2 mg (▲, n = 5), 0.4 mg (□, n = 4), and 1.0 mg (■, n = 5) CCK all produced levels of protein and acid phosphatase during the subsequent 60 min which were significantly higher than control infusions (●, n = 6). Infusion of control Ringer did not produce protein or acid phosphatase levels significantly different from controls where no infusion was given (Fig. 3).

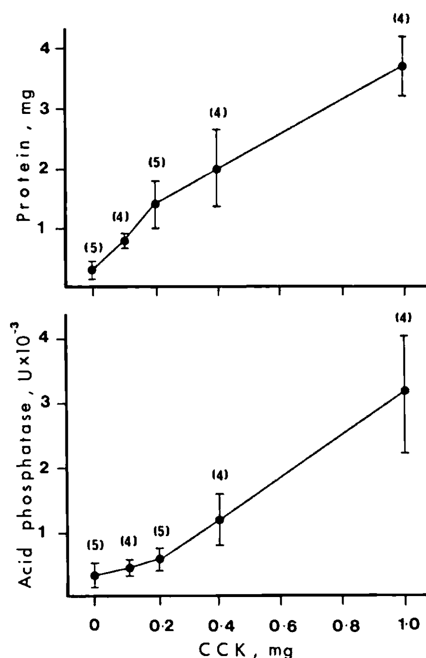

FIG. 5. Effect of gastric production of protein and acid phosphatase of infusion of Sigma CCK (0.1–1.0 mg). Response to each dose is estimated by calculating the total amount of protein and acid phosphatase produced during the 60-min postinfusion period. Mean ± SE (number of animals in parentheses). In both instances an approximately linear relationship between dose and response exists.

Effect of Hormones on Protein and Acid Phosphatase Levels

Infusion of Sigma CCK, at all doses, produced a significant elevation of perfusate protein and acid phosphatase, with maximal levels occurring 10–20 min postinfusion (Fig. 4). Recovery occurred within 60 min with doses of 0.1, 0.2, and 0.4 mg, but 1.0 mg produced elevated levels over the entire 60-min sampling period.

Figure 5 shows an approximately linear relationship between both acid phosphatase and protein output, and the doses of Sigma CCK infused. Total gastric output was estimated by calculating the amounts of protein or acid phosphatase present in all the samples of perfusate collected during the 60-min postinfusion period.

Cholecystokinin-octapeptide (Peninsula) (0.1 mg) produced a considerably larger response than did the Sigma CCK at the same dose (Fig. 6). The maximal protein level occurred after 20 min (25 ± 3.7 mg/dl) with a recovery to 6.0 ± 1.2 mg/dl after 60 min. The maximal acid phosphatase level occurred after 10 min. (7.0 ± 1.4 units/liter) with a recovery to 0.4 ± 0.4 units/liter. Figure 6 also shows that infusion of 0.1 mg of synthetic porcine secretin produced levels of protein and acid phosphatase over the subsequent 60 min which were not significantly different from control values.

DISCUSSION

The results show that porcine CCK and CCK-8 stimulate gastric release of acid phosphatase and probably other enzymes measured as Lowry protein (Figs. 4, 6). In mammals, two of the major physiological

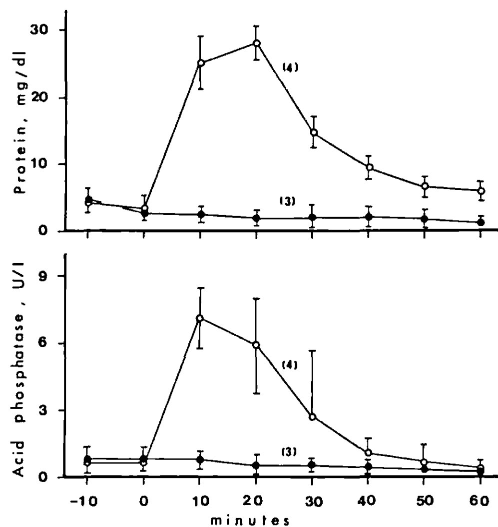

FIG. 6. Effect of 0.1 mg CCK-8 (○) and 0.1 mg secretin (●) on gastric protein and acid phosphatase levels over the 60-min postinfusion period. Mean ± SE (number of animals in parentheses). CCK-8 produced a significant elevation of protein levels above control values (Fig. 3) over the 60-min postinfusion period, maximal levels occurring after 10 min (25 ± 3.7 mg/dl). CCK 8 also produced a significant elevation of acid phosphatase levels, maximal levels occurring after 10 min (7.02 ± 1.4 U/liter). Secretin caused no significant elevation of either protein or acid phosphatase levels over the 60-min postinfusion period.

actions of CCK are the stimulation of pancreatic enzyme secretion and initiation of gall bladder contraction: the ecbolic or pancreozymin effect and the cholecystokinetic effect, respectively. In *Styela*, CCK stimulates only enzyme secretion, since these animals do not possess a gall bladder homologue, or any other gut-associated smooth muscle. Vigna and Gorbman (1979) have similarly found that CCK may act in a "pancreozymin" fashion on enzyme secretion in the hagfish *Eptatretus stouti*, and have demonstrated that porcine CCK-33 induces intestinal lipase secretion, but not gall bladder contraction. However, acid–alcohol extracts of the intestine of the hagfish *Myxine glutinosa* can cause guinea pig gall bladder contraction (Nilsson, 1973), and intestinal extracts of the lampreys *Lampetra fluviatilis* and *Petromyzon marinus* have a CCK-like effect on mammalian gall bladder and pancreas (Barrington, 1971; Barrington and Dockray, 1970). Dockray (1977), has suggested that regulation of gall bladder contraction may have evolved before the control of intestinal enzyme secretion in the Agnatha. However, our results and those of Vigna and Gorbman (1979) both appear to support the opposite contention.

The present results indicate that secretory cells in the gastric epithelium of *Styela*, probably the zymogen cells (Thorndyke, 1977), have, located on their basal plasma membranes, a class of receptors sensitive to porcine CCK. Furthermore it seems likely that the receptors are part of a cytoplasmic system controlling the release of secretory products.

However, immunofluorescence studies (Bevis and Thorndyke, 1979; Thorndyke and Bevis, 1980) have failed to show the presence of a CCK-like peptide in the gastric endocrine cells of *Styela*. Immunofluorescence studies with CCK/gastrin C-terminal tetrapeptide as well as antisera to human gastrin have proved negative (Bevis and Thorndyke, 1979; Thorndyke and Bevis, 1980), whereas similar tests with antisera prepared against secretin have proved positive (Bevis and Thorndyke, 1979). We have so far been unable to obtain antisera to CCK-33 or CCK-8.

Thus the present results suggest the control of gastric secretory activity by a CCK-like factor rather than the secretin-like factor indicated by the immunocytochemical results. Despite this apparent contradiction, we suggest that the cap endocrine cells release a peptide which may act on neighbouring zymogen cells in a paracrine fashion (Fig. 1).

Weinstein (1972) has suggested, on theoretical grounds, that both the secretin/glucagon/VIP family and the gastrin/CCK family arose from a single parent molecule which he termed prosecgastrin. Holmquist *et al.* (1979) have suggested that in lampreys small CCK/gastrin- and glucagon/

secretin-like peptides may already be produced separately, while Van Noorden and Pearse (1974, 1976) have demonstrated that a single cell type, found in the gut of lamprey and the more primitive *Branchiostoma*, has the ability to react with both anti-glucagon and anti-gastrin sera. They suggest that this double immunoreactivity is due to the presence of a prosecgastrin type of parent hormone. We suggest that this may be the condition of *Styela*, where cap endocrine cells contain a prosecgastrin molecule. It may be that in such a large molecule, only the secretin-like region is accessible for immunostaining, or equally, that the CCK-tetrapeptide antiserum that we have used is effective only against vertebrate CCK and not the *Styela* prosecgastrin molecule. This is consistent with observations that pentagastrin (Peptavlon) does not induce gastric secretion in *Styela* (Thorndyke and Bevis, 1980), and it is clearly of importance to test whether or not other CCK and CCK-related peptide antisera produce positive immunofluorescence.

The large doses of CCK which were required to produce a significant response suggest that although CCK-like, the peptide involved is not identical to porcine CCK. The possibility that CCK-related peptides such as caerulein and phyllocaerulein are biologically active is certainly relevant, and will require testing.

Further immunocytochemical and physiological work in this field would be of considerable value both in determining the nature of the suggested *Styela* prosecgastrin, and the nature of the CCK-initiated enzyme release. Such a study is currently being undertaken in this laboratory.

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Endocrine Cells in the Gut of the Ascidian *Styela clava*

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Summary. Ultrastructural studies have shown the presence of two types of granulated endocrine cell in the gut of *Styela clava*. Type I, which occurs in the stomach and intestine contains small irregular granules, each with a distinct halo. Type II, found only in the oesophagus contains larger rounded granules, often with little or no halo. The characteristics of these two cell types are compared with those of endocrine cells found in the digestive tracts of other protochordates and discussed with special reference to the evolution of gastrointestinal endocrine cells in vertebrates.

Key words: Endocrine cells – Ascidian (*Styela clava*) – Gut epithelium – Electron microscopy.

Introduction

The gastrointestinal mucosa in higher vertebrates is characterised by the presence of a wide range of endocrine cell types (Andrew 1976a, b; Capella et al., 1969; Forssmann, 1970; Polak et al., 1976; Solcia et al., 1975) and ultrastructurally they have been classified according to their granule type and size (Andrew, 1976; Solcia et al., 1975; Polak et al., 1976). A number of other cytochemical and immunocytochemical characteristics are shared by these cells which produce polypeptide hormones and/or biogenic amines and have been used by Pearse in the development of the so called APUD concept (Pearse, 1968, 1969). These features are thought to be connected with the common origin of the cells in neuroectodermal tissue (Pearse, 1969; Pearse et al., 1973). Observations on the digestive epithelia of

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lower vertebrates have indicated the presence of cells with similar characteristics (Ostberg et al., 1976; Van Noorden and Pearse, 1974; Van Noorden et al., 1972).

Limited studies on invertebrates have suggested that the gut in these animals may also have an endocrine capacity (Boquist et al., 1971; Davidson et al., 1971; Falkmer and Ostberg, 1976).

In the protochordates there are a few reports available on the occurrence of amines and chromaffin cells (Erspamer, 1946; Erspamer and Asero, 1952; Gerzeli, 1961, 1963; Welsh and Loveland, 1968). More recent ultrastructural and histochemical work has shown the presence of granulated endocrine-like cells in the gut of several Ascidian species (Burighel and Milanesi, 1975; Fritsch and Sprang, 1977; Thorndyke, 1977), while other workers, (Van Noorden and Pearse, 1976) have described a number of granulated cell types in the gastrointestinal epithelium of amphioxus (*Branchiostoma lanceolatum*). The present report describes the occurrence of similar granulated endocrine-like cells in the gut of *Styela clava*.

Materials and Methods

Specimens of *Styela clava* collected in the Portsmouth area were transported to London in aerated sea water and transferred to circulating sea water aquaria at 10° C. The oesophagus, stomach and intestine were rapidly excised into cold (4° C) 3 % glutaraldehyde, buffered with 0.1 M sodium cacodylate (pH 7.2) in sea water. Following dissection, small fragments of each tissue (1 mm^3) were transferred to aliquots of the same fixative for a total of 2 h. Post fixation was for 1 h in 1 % OsO_4 in the same buffer followed by dehydration in graded ethanols and embedding in TAAB epoxy resin. Thin sections (pale gold-silver) were cut on a Cambridge Huxley Automatic microtome, counterstained with uranyl acetate and lead citrate and examined in an AEI EM6B or Corinth 275 microscope.

Results

Granulated, endocrine-like cells may be found at all levels in the post pharyngeal digestive epithelium of *Styela*, although they are most commonly seen in the stomach and oesophagus (Fig. 1a–d). The gastric epithelium in *Styela* comprises a series of ridges, the side walls of which are populated by both absorptive and protein secreting cells, while the cap of each ridge is composed of mucous cells (Thorndyke, 1977). It is only in this latter region that the gastric endocrine cells are found (Fig. 1a, b). The oesophagus and intestine do not show this type of regional specialization and endocrine cells are evenly distributed amongst the epithelial mucous cells (Fig. 1c, d).

Throughout the gut the endocrine cells usually occupy a basal position (Fig. 1a, d), although there are indications that this may be due to the plane of section, since on occasions in both stomach and oesophagus the cells take on a more slender, columnar, appearance (Fig. 2a, b).

The granules in the gastric and intestinal endocrine cells (Type I) are small, electron dense and rather irregular in profile, with a distinct halo separating granules from membrane (Figs. 1b, 3a). They are scattered throughout the cytoplasm and range from 50–155 nm in diameter (90 nm average). The remainder of the cytoplasm is filled with a largely vesicular smooth endoplasmic reticulum, interspersed with free ribosomes and mitochondria (Figs. 1b, 3a). The granules in

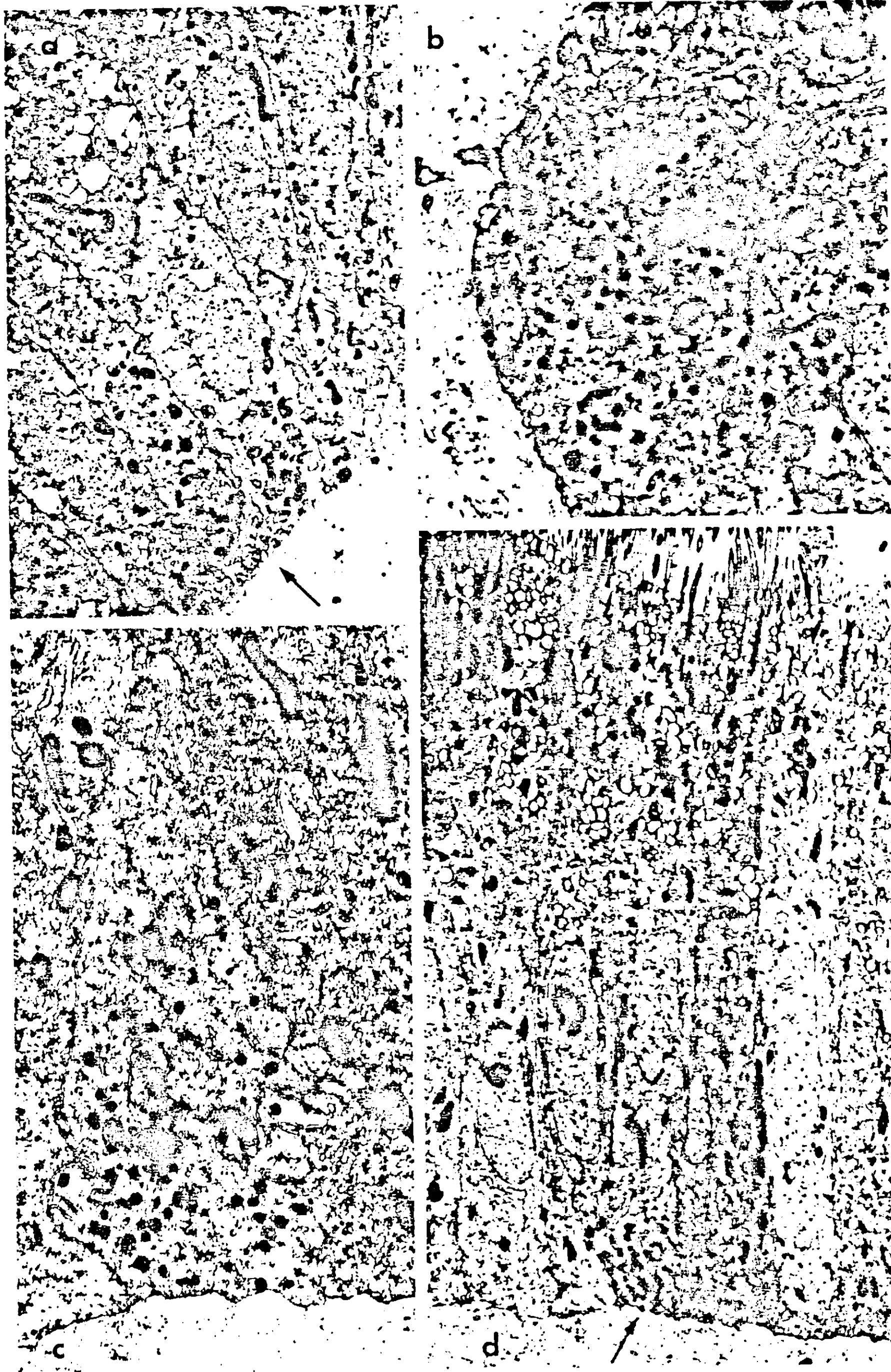

Fig. 1. a and b Gastric endocrine cells showing typical position on basement membrane. a ×4000. b ×18,000. **c** and **d** Oesophageal endocrine cells sited basally between columnar mucous cells. c ×13,000. d ×4000

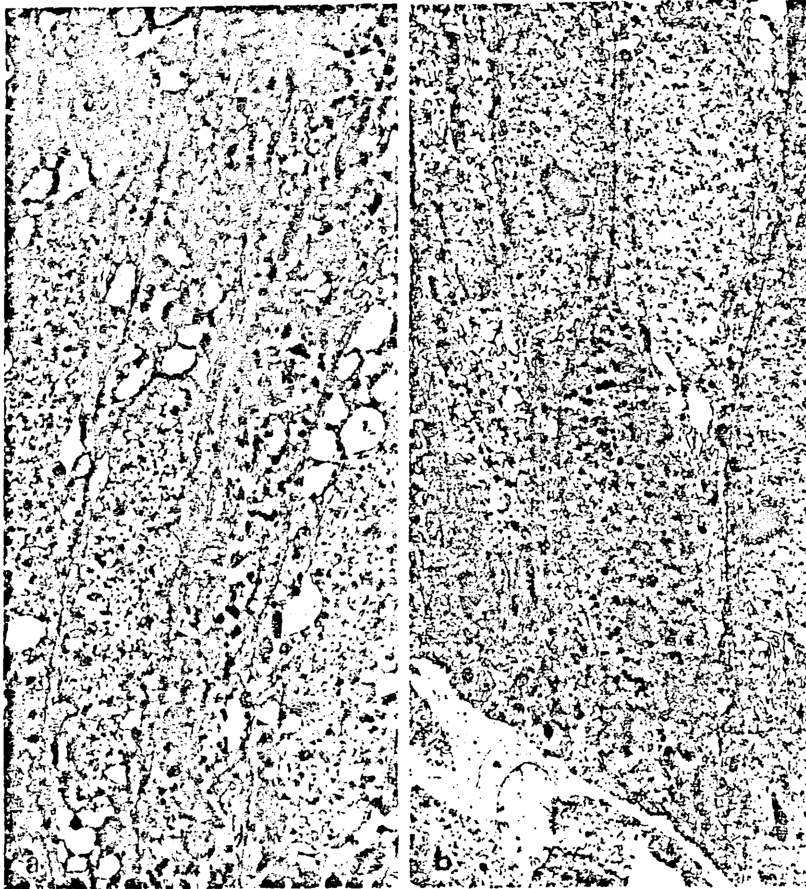

Fig. 2 a and b. Electron micrographs of the more elongated columnar endocrine cells occasionally found in the stomach (a) and oesophagus (b). a ×6300, b ×5400

the oesophageal endocrine cells (Type II) on the other hand are more regular in shape with a very narrow, or at times, non-existent halo and vary between 100–175 nm in diameter (average 130 nm) (Figs. 1c, 3b).

Discussion

This present report shows that the gastrointestinal epithelium of *Styela*, like other protochordates (Burighel and Milanesi, 1975; Kataoka and Fujita, 1974; Fritsch and Sprang, 1977; Van Noorden and Pearse, 1974), carries a population of granulated endocrine-like cells which possess some of the features exhibited by the polypeptide hormone producing members of the APUD series in vertebrates (Ostberg et al., 1976; Pearse, 1968, 1969, 1974; Solcia et al., 1975; Van Noorden and Pearse, 1974). Reports on Ascidians (Burighel and Milanesi, 1975; Fritsch and

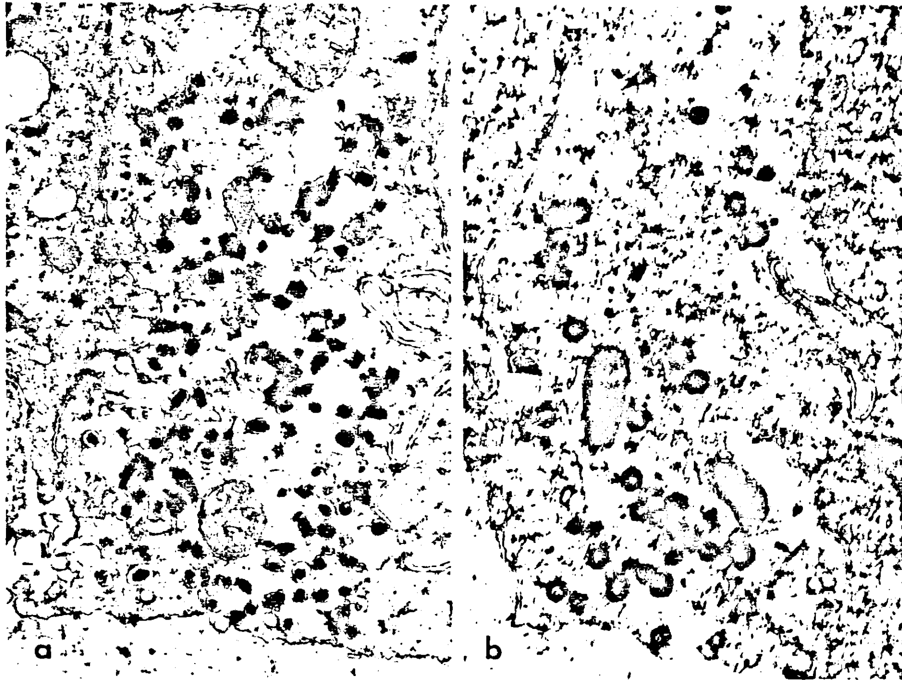

Fig. 3. a Details of gastric endocrine cell showing granules with irregular, electron-dense core, surrounded by a distinct halo. × 20,000. **b** Oesophageal endocrine cells, the granule cores have a more regular outline and the halo is narrow and indistinct. × 24,500

Sprang, 1977) cannot, on ultrastructural evidence, distinguish different types of endocrine cell; although the observations of Fritsch (1976) suggest that more than one type may occur in *Ciona*. Similarly recent work on cyclostomes describes only a single gut endocrine cell type (Ostberg et al., 1976; Van Noorden and Pearse, 1974).

In sharp contrast to this, observations on the digestive epithelium of *Branchiostoma* (Kataoka and Fujita, 1974; Van Noorden and Pearse, 1976) find at least two cell types, perhaps even three or four, classified according to their ultrastructural appearance. Thus in *Branchiostoma*, type I cells have dense, irregular granules with an obvious halo and an average diameter of 140 nm. Type III are similar but with eccentric cores and an average diameter of only 60 nm (Van Noorden and Pearse, 1976). The type II cell granules in this species are more rounded with a narrow or non-existent halo and a mean diameter of 170 nm (Van Noorden and Pearse, 1976). Our observations indicate similarities between *Styela* and *Branchiostoma*, each with more than one cell type classified by the size and appearance of granules (Figs. 1b, c, 3a, b). The type I cells found in the stomach and intestine of *Styela* fall somewhere between the types I and III of *Branchiostoma*, while the Styela type II seem comparable to *Branchiostoma* type II. Clearly there are some differences in granule size range between these two species, as there is between *Styela* and *Ciona* (Fritsch and Sprang, 1977) where the single endocrine cell type approaches *Styela* type I. However, this only serves to emphasize the problems

associated with this work, where the ultrastructural appearance of granules is so markedly affected by the method of fixation employed (Mortensen and Morris, 1977). The most that can be said for *Styela*, where the same fixation routine was used throughout, is that there are two types of granulated cell, types I and II, which bear some resemblance, albeit superficial, to the type I/III and type II respectively in *Branchiostoma*.

The final outcome of this preliminary classification depends on a number of other, cytochemical and immunocytochemical, tests (Pearse, 1968, 1969; Van Noorden et al., 1972). Following such tests, *Branchiostoma* type I cells have been equated to argyrophilic, glucagon/gastrin immunoreactive cells and type III to biogenic amine producing cells (Van Noorden and Pearse, 1976). Initial observations on *Styela* type I cells (Bevis and Thorndyke, unpublished observations) indicate the production of biogenic amines. This agrees with the histological findings of Gerzeli (1963) and Fritsch (1976) in *Ciona* and with other biochemical investigations (Erspamer and Asero, 1952; Welsh and Loveland, 1968). The localization of a polypeptide hormone-like factor awaits final confirmation. An exploratory analysis of *Styela* type II cells suggests they are aldehyde fuchsin positive and possibly produce an insulin-like factor (Bevis and Thorndyke, 1978). These features are in agreement with the characteristics of the similar type II cell in *Branchiostoma* (Van Noorden and Pearse, 1976).

Thus, in *Styela* there appears to be at least two distinct endocrine cell types in the gastrointestinal epithelium. It may also be that each cell type in turn produces more than one factor, as suggested for the endocrine cells in *Ciona* (Fritsch and Sprang, 1977) and *Branchiostoma* (Van Noorden and Pearse, 1976). The nature of the product or products, awaits further investigation, although on present evidence it seems probable that it is a polypeptide hormone and/or biogenic amine.

Furthermore it is still far from clear as to whether these substances are released as hormones into the blood system, as postulated for *Branchiostoma* (Kataoka and Fujita, 1974). Similarly it is not known whether they have any normal physiological role in the life of the animal.

Whatever the answers to these questions are, there can be no serious doubt that the present situation in Ascidians with regard to gut endocrine cells is of crucial significance to our understanding of the evolution of gastrointestinal endocrine cells in vertebrates.

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